Delineating the Cytokine Profile of Encephalitogenic T cells

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Summary

Multiple sclerosis (MS) is the most common inflammatory disease of the central nervous system (CNS) and its animal model experimental autoimmune encephalomyelitis (EAE) is mediated by the actions of auto-reactive encephalitogenic T_H cells. While T_H1 cells were long considered to be the major pathogenic population, the discovery that IL-23 and not the T_H1 inducing cytokine IL-12 is vital for EAE development initiated a major paradigm shift with regards to the role of T_H1 cells in inflammation. IL-23 supports the expansion of IL-17 secreting T_H cells, which are now considered to be the pathogenic population mediating autoimmunity as IL-17 expression by T cells correlates closely with an autoimmune pathogenic phenotype and this polarization pattern was termed T_H17. However, IL-17A in contrast to IL-23 does not appear to be an absolute requirement for the development of autoimmune disease and other features appear to be inherent to this pathogenic T_H cell population.

We identified IL-22 as well as IL-17F to be induced by IL-23 in encephalitogenic $CD4^+$ T cells and could show that both cytokines are specifically secreted by T_H17 cells implicating them as additional encephalitogenic cytokines. To determine the role of these cytokines in autoimmune inflammation, we generated IL-$22^{-/-}$ and IL-$17F^{-/-}$ mice. In contrast to the notion that genes associated with CNS-invading T cells imply their pathogenic function, we found IL-$22^{-/-}$ as well as IL-$17F^{-/-}$ mice to be fully susceptible to EAE.

Taken together, we show that self-reactive T cells coexpress IL-17A, IL-22 and IL-17F, but that neither IL-22 nor IL-17F are involved in autoimmune pathogenesis of EAE.

On human blood-brain barrier endothelial cells in multiple sclerosis lesions, we could demonstrate the expression of IL-17 and IL-22 receptors and show that IL-17 and IL-22 disrupt BBB tight junctions *in vitro* and *in vivo*. We could further detect numerous IL-17 or IL-22 expressing T cells in highly infiltrated MS lesions and observed that these cells produced cytolytic enzymes such as granzyme B. Granzyme B^+ T_H17 cells had the capacity to kill human fetal neuron–enriched cultures and showed considerable cytolytic activity as compared to unactivated T cells.

Therefore, we conclude that additional factors expressed by T_H17 cells or induced by IL-23 – distinct from IL-17A, IL-17F and IL-22 – exist that play a role in autoimmune inflammation.

Zusammenfassung

Multiple Sklerose (MS) ist die am weitesten verbreitete inflammatorische Autoimmunerkrankung des Zentralnervensystems (ZNS) und das entprechende Tiermodell experimentelle autoimmune Enzephalomyelitis (EAE) wird durch autoreaktive enzephalitogene T_H Zellen hervorgerufen. Lange wurden die pathogenen Eigenschaften der T_H1-Zellpopulation zugeschrieben, doch die Entdeckung, dass nicht das T_H1 induzierende Zytokin IL-12, sondern IL-23 für die Entwicklung von EAE verantwortlich ist, führte zu einem Paradigmenwechsel bezüglich der Rolle von T_H1 Zellen in entzündlichen Erkrankungen. IL-23 bewirkt die Expansion von IL-17 exprimierenden T_H Zellen, die nun als pathogene und Autoimmunität vermittelnde Zellpopulation angesehen werden, da die IL-17 Expression eng mit einem autoimmunen Phänotyp assoziiert ist. Diese IL-17 sekretierenden T-Zellen werden als T_H17 Zellen bezeichnet. Im Gegensatz zu IL-23 scheint IL-17A allerdings keine absolute Voraussetzung für die Entwicklung von Autoimmunerkrankungen zu sein und T_H17 Zellen scheinen weitere Moleküle mit proinflammatorischen Eigenschaften zu exprimieren.

Wir konnten zeigen, dass IL-22 und IL-17F durch IL-23 induziert, von enzephalitogenen $CD4^+$ T-Zellen exprimiert und als weitere T_H17 Zytokine anzusehen sind. Um die Rolle dieser Zytokine in Autoimmunentzündungen näher zu untersuchen, haben wir IL-$22^{-/-}$ und IL-$17F^{-/-}$ Mäuse hergestellt. Im Gegensatz zur Annahme, dass mit ZNS infiltrierenden T-Zellen assoziierte Gene eine pathogene Funktion bedeuten, entwickelten IL-$22^{-/-}$ und IL-$17F^{-/-}$ Mäuse normale EAE. Zusammenfassend können wir zeigen, dass autoimmune T-Zellen IL-17A, IL-17F und IL-22 koexprimieren, allerdings weder IL-22 noch IL-17F in der Entwicklung von EAE eine Rolle spielen.

Wir konnten die Rezeptoren für IL-17 und IL-22 auf humanen Endothelzellen der Bluthirnschranke in MS Läsionen nachweisen und zeigen, dass IL-17 und IL-22 die Zellverbindungen der Bluthirnschranke zerstören – sowohl *in vitro* als auch *in vivo*. Desweiteren konnten wir eine Vielzahl von IL-17 und IL-22 exprimierenden T-Zellen in infiltrierten MS Läsionen detektieren und feststellen, dass diese zytolytische Enzyme wie Granzym B exprimieren. Granzym B^+ T_H17 Zellen waren in der Lage, neuron-angereicherte Zellkulturen zu lysieren und zeigten im Vergleich zu nicht aktivierten T-Zellen eine beachtliche zytolytische Aktivität.

Insgesamt können wir den Schluss ziehen, dass weitere Faktoren – exprimiert von T_H17 Zellen bzw. induziert durch IL-23 – existieren, die eine wichtige Rolle in der Entwicklung von Autoimmunentzündungen spielen.

Introduction

The immune system

The immune system is a complex network of organs and cells that protect the body against pathogens or infectious agents, such as viruses, bacteria, fungi and other parasites. It can be divided into two distinct but intertwined systems: the innate and the adaptive immune system. Innate immunity is present in all vertebrates as well as in plants and insects and allows the recognition of pathogens and microbes through preformed receptors (1). Adaptive immunity is a feature of higher vertebrates which allows the recognition of foreign structures through receptors which are molded in primary lymphoid organs by means of somatic recombination. Mature lymphocytes, namely T- and B-cells, carry such recombined receptors. Adaptive immunity furthermore has developed a vital feature of immunity - the capacity to “remember” formerly encountered antigens. While innate immunity originally was considered a primitive predecessor of adaptive immunity, it has become clear that the development of adaptive immune responses absolutely requires the assistance of innate immunity and to some extend vice versa (2).

Innate immunity

The innate immune response provides a first line of defense for the organism in controlling infections in the first days before an initial adaptive immune response takes place. The components of the innate immune system are natural killer (NK) cells, γ/δ T cells, polymorphonuclear cells (PMNs) and professional antigen presenting cells (APCs) like monocytes/macrophages and dendritic cells (DCs). Its function is based on the recognition of pathogen-associated molecular patterns (PAMPs), which are typically nucleic acids or conserved components of cell wall structures from microorganisms. PAMPs are recognized by germ-line encoded receptors known as pattern recognition receptors (PRR) (3),which include membrane-bound receptors on the cell surface like Nucleotide-binding oligomerization domain proteins (NOD) recognizing components of peptidoglycan and mediate intracellular recognition of bacteria (4) as well as soluble proteins secreted into the blood like mannose-binding lectins (MBL) (5), C-reactive protein (CRP) (6) or complement (7). The most important type of membrane-bound PRRs are the toll-like receptors (TLRs), which are expressed on DCs and macrophages (1). Nowadays, 11 different TLRs have been characterized in mice (8). Among them bacterial CpG DNA is recognized by TLR9, cell wall components of Gram-positive bacteria by TLR2 and TLR4 functions as the pattern of recognition receptor for LPS. Engagement of TLRs promotes

the engulfment and destruction of microorganisms and leads to increased phagocytic activity, release of proinflammatory factors and secretion of chemokines and cytokines. Most importantly, APC activation and maturation is a requirement to activate and drive adaptive immunity (2).

Adaptive immunity

The adaptive immune system recognizes foreign structures through highly specific receptors which are generated by somatic recombination and therefore offers a more precise and efficient recognition of pathogens as compared to innate immunity. Most importantly, adaptive immunity generates immunological memory, which enables rapid immune activation upon reinfection. The adaptive immune response includes B-cell mediated humoral immunity and T cell mediated cellular immunity.

B-cells mature in the bone marrow and express a unique B-cell receptor (BCR), which is a membrane bound antibody molecule. Antibodies are made up of a variable region which

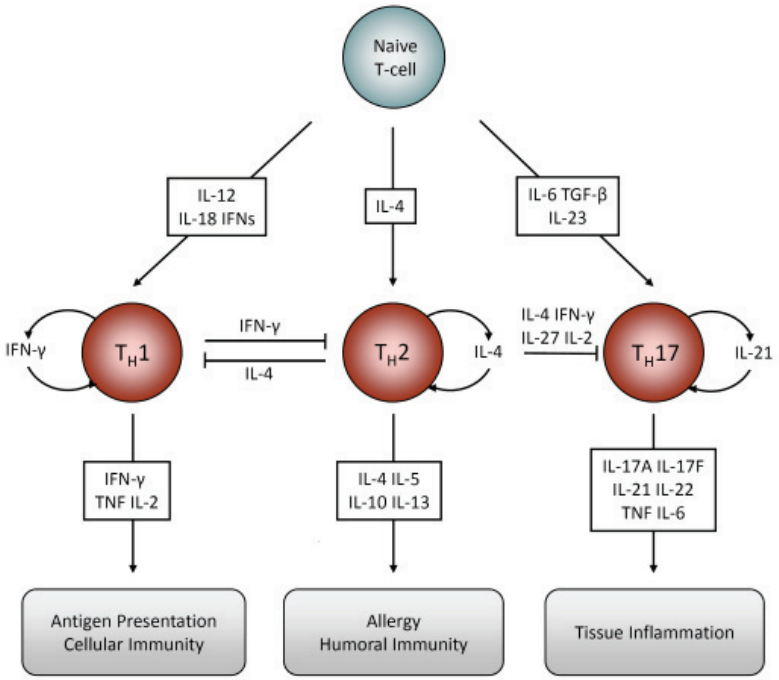

Figure 1: Schematic illustration of the induction and effects of T_H1, T_H2 and T_H17 cells.
The cytokines secreted by antigen presenting cells during T cell activation direct the differentiation of naïve $CD4^+$ T cells towards one of different effector T_H cell lineages which secrete characteristic cytokines and exert distinct functions.The classical T_H1 polarizing factors include type I and II IFNs, IL-12, IL-18 and IFN-γ produced by T_H1 cells is important in the regulation of antigen presentation and cellular immunity, while the T_H2-cell cytokines regulate B-cell responses and are crucial mediators of allergic diseases. T_H17 cells, which are triggered by TGF-β, IL-6 and IL-23 express IL-17, IL-17F, IL-21 and IL-22 and mediate inflammatory responses. IL: interleukin, IFN: interferon, TNF: tumor necrosis factor, TGF-β: transforming growth factor-β.

includes the highly specific antigen (Ag)-binding site and a constant region, which defines the different classes of antibodies. In mice and humans the different isotypes of antibodies are IgM, IgG, IgA, IgD and IgE, which differ in structure, effector function, localization and serum concentration. Antibodies protect the host by neutralization through binding to structures on the surface of pathogens, by opsonization which is achieved by coating the pathogen and promotes phagocytosis and subsequent elimination of the pathogen or by activation of the complement system. The complement system strongly enhances opsonization, but can also lead to direct killing of certain bacterial cells by inserting pores in their membranes. The type of effector mechanism depends in part on the antibody isotype. Isotype switching occurs after B-cell activation and leads to production of different classes of antibodies. B-cell activation can be T cell dependent or T cell independent. Most antigens are T cell dependent, in which case Ag crosslinking the BCR provides the first signal necessary for B-cell activation and the second signal comes from co-stimulation provided by a T helper (T_H) cell, which produces cytokines that trigger B-cell proliferation and differentiation into an antibody secreting plasma cell. Isotype switching and memory cell generation occur in response to T cell dependent antigens. T cell independent antigens often comprise structural epitopes like repeating carbohydrate epitopes that can stimulate B-cells without T_H cell help (9).

T cells are also generated in the bone marrow but migrate to the thymus for maturation during which they undergo a positive selection for the recognition of self major histocompatibility complex (MHC) molecules, followed by a negative selection. During negative selection, T cells expressing a T cell receptor (TCR) recognizing self-peptides are eliminated to prevent an immune response against the bodies own tissues. T cells that survived positive as well as negative selection in the thymus circle in a naïve stage through the body via the blood and lymph streams. T lymphocytes only recognize processed peptide antigens presented on MHC molecules (10) - cytotoxic $CD8^+$ T cells react to peptide bound on MHC class I molecules which are expressed by virtually all cells of the body and $CD4^+$ T_H cells recognize MHC class II bound peptide. MHC class II molecules are only expressed by professional APCs and contact between an APC providing a suitable MHC/peptide complex (signal 1) and an additional costimulatory signal (signal 2) is required for the activation of the T cell, a process called priming. Therefore, APCs can be seen as the link between innate and adaptive immunity. Two of the best characterized costimulatory molecules expressed by APCs are CD80 (B7.1) and CD86 (B7.2) which interact with CD28 or CTLA-4 expressed on T cells, providing a stimulatory or inhibitory signal, respectively. CTLA-4 is only expressed upon activation of T cells and therefore serves to contain the immune response. Activation of T cells without co-stimulation may lead to T cell anergy or deletion. T cells also provide stimulatory signals for APCs through expression of CD40L

which interacts with its receptor CD40 on APCs (11). CD40 signaling leads to cell activation and survival and further upregulation of costimulatory molecules.
The cytokines secreted by APCs are referred to as a third signal during T cell activation and greatly influence the outcome of the immune response as T_H cells can differentiate into one of several subtypes with distinct functions depending on the cytokine environment (**Fig. 1**).
The classical T_H1 polarizing factors include type I and II interferons (IFNs), interleukin (IL-) 12, IL-18 and T_H1 cells secrete IFN-γ and IL-2 (12) leading to activation of macrophages and $CD8^+$ T cells. In turn, in the absence of T_H1 cytokines and through IL-4 polarized T_H2 cells a humoral immune response is supported by secretion of IL-4 (13) which leads to an activation of antibody secreting B-cells and the complement system. This classical T_H1/ T_H2 paradigm has been recently updated after additional effector T cell types have been identified, i.e. IL-17 producing T_H17 cells which polarization is mediated by TGF-β, IL-6 and IL-23 (14,15,65,79,195). Moreover, additional $CD4^+$ subsets such as T_H3 cells, T regulatory type 1 (T_R1) cells, TGF-β induced regulatory T cells and follicular helper T (T_{FH}) cells have been characterized (16). Regulatory T cells (T_{reg}) are important in maintaining peripheral tolerance and limiting extensive immune response via direct cell-cell contacts and/or by the production of suppressive cytokines like IL-10 and TGF-β.
Activated $CD8^+$ cytotoxic T lymphocytes (CTLs) are able to destroy their target cells through induction of apoptosis, either using perforin and granzymes (17) or Fas-ligand interaction (18,19). In the first pathway, cytoplasmic granule toxins — predominantly the membrane-disrupting protein perforin — and a family of structurally related serine proteases (granzymes) with various substrate specificities are secreted by exocytosis and together induce apoptosis of the target cell. The existence of $CD4^+$ CTLs, which are able to perform perforin and granzyme-mediated killing, has also been reported (20).

Autoimmunity

Autoimmunity is caused by an adaptive immune response against “self” antigen. Normally, lypmhocytes expressing an antigen receptor that recognizes self antigen are eliminated during negative selection. As soon as a developing lymphocyte expresses its antigen receptor on the cell surface, its antigen-recognition properties are tested. Specificity and affinity towards molecules present in the immediate environment, which is the bone marrow and fetal liver for B-cells and the thymus for T cells, are tested.
Strong autoantigens that crosslink the BCR lead to elimination of these autoreactive B-cells through cell death or apoptosis (21,22). Furthermore, the B-cell can be rescued

by rearranging the BCR and creating a self-tolerant BCR by receptor-editing (23). Weak autoantigens can lead to anergy of the B-cell which then dies relatively soon due to missing survival signals from T cells. Some autoreactive B-cells however, which did not encounter their antigen during development as it is not present in the compartments of lymphopoisis, might be released into the periphery and lead to autoimmunity.

T cells expressing a TCR that recognizes a self peptide in the context of a MHC molecule are eliminated during negative selection in the thymus – a mechanism known as central tolerance. It is clear however, that thymic selection is not a perfect process and that autoreactive T cells are frequently released into the periphery. One reason for this is that not all peripheral self-antigens are expressed within the thymus and those that are may not always be present at sufficient levels to cause deletion. To keep these potentially harmful T cells in check, several control mechanisms exist that are collectively referred to as peripheral tolerance. Potentially harmful cells can either be rendered anergic (i.e. functionally unresponsive), deleted by apoptosis, or suppressed by T_{reg} cells within secondary lymphoid or non-lymphoid organs (24-26).

If, however, all control mechanisms fail, the immune system can attack the body's own tissues and initiate an autoimmune disease. Autoimmune diseases can be systemic and directed against various organs or organ-specific. The majority of systemic autoimmune diseases like systemic lupus erythematusus (SLE) result from humoral immune responses and the production of autoantibodies, whereas organ-specific autoimmune diseases like Insulin-dependent (Type I) diabetes mellitus where pancreatic β-cells are the target, rheumatoid arthritis (RA) in which the joint synovium is attacked or multiple sclerosis are generally believed to be cell mediated.

Multiple Sclerosis

Multiple sclerosis (MS) is a complex demyelinating disease associated with inflammation in the central nervous system (CNS) and is thought to be driven by an autoimmune process. It develops in young adults and common features are paralysis, sensory disturbances, lack of coordination and visual impairment (27), whereas the clinical manifestations of MS among affected individuals are extremely variable. Pathologically, MS is characterized by lesions or plaques found in the CNS white matter, which result from focal loss of myelin and are localized throughout the CNS, but predominantly reside within the periventricular regions, optic nerves, brain stem and spinal cord. The sites of active demyelination are characterized by infiltration of MHC class II expressing macrophages, B-cells and activated $CD4^+$ T cells secreting distinct cytokines (28). The activation of autoreactive $CD4^+$ T cells against components of the myelin sheath and their differentiation into a pathogenic phenotype are considered crucial events in the initial phase, whereas further damage

of the CNS is most likely mediated by other components of the immune system, such as antibodies, complement, $CD8^+$ T cells and factors produced by innate immune cells. At the same time, a degenerative process distinct from the recurrent inflammation is thought to be responsible for the progressive loss of neural cells and neurological functions (29).
Despite intensive scientific research, the etiology of MS remains largely unknown, but is widely accepted to be an autoimmune disease which susceptibility is influenced by both genetic and exogenous factors.

Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) serves as an animal model of MS. It can be induced in susceptible rodents by immunization with myelin antigens such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP) or by adoptively transferring an expanded population of myelin-reactive encephalitogenic $CD4^+$ T cells (30-32). EAE cannot be transferred by antibodies and has so far only been reported 2 times to be inducible by the transfer of $CD8^+$ cells (33,34), emphasizing the important role of $CD4^+$ T cells.
The sequence of immunopathological events leading to EAE can be divided into an initial T cell priming and activation phase and a subsequent recruitment and effector phase (35). After immunization with myelin antigens, these are taken up and presented by professional APCs in secondary lymphoid organs which results in the activation and expansion of auto-aggressive, myelin-reactive T cells. In the EAE model, adjuvants such as complete Freund's or microbial DNA containing CpG motives are generally used to break immune tolerance against self-structures and initiate APC activation and maturation. Immunization with soluble Ag does not suffice to induce autoimmunity. After successful priming, activated myelin-reactive T cells migrate to the CNS where they reencounter their Ag in the context of an APC (36) followed by blood brain barrier (BBB) permeabilization allowing them to migrate deeper into the CNS parenchyma (37). The production of proinflammatory cytokines such as tumor necrosis factor (TNF) and IFN-γ, leads to the up-regulation of MHC class II molecules on APCs and also of adhesion molecules on the BBB endothelium. This will facilitate the further influx and activation of cells, thus contributing to the amplification of the inflammatory immune response ultimately leading to demyelination. Other than disease induction, the steps involved in the pathogenesis of EAE and MS are thought to be similar.

The blood brain barrier

The CNS has long been considered to be an immune privileged organ. The concept of immune privilege was drawn upon the observation, that allografts showed prolonged survival in the CNS while being rapidly rejected by the immune system when grafted into other sites, such as skin (37a). Due to the lack of draining lyphatics and the presence of the BBB, the CNS was regarded as being immunologically inert and completely isolated from the peripheral immune system. However, recent data revealing types of cellular immune responses that occur within the CNS proved that it is neither isolated nor passive in its interaction with the immune system. Peripheral immune cells can cross the intact BBB, glia and neurons actively regulate macrophage and lymphocyte responses and microglial cells are immunocompetent. It was suggested, that specific types of immune responses are even required for the maintenance of normal CNS function and the induction of regeneration (38-40). Therefore, immune privilege is rather the sum of CNS driven mechanisms actively regulating T cell responses within the CNS than a complete immune isolation.

In the old view of immune privilege as immune isolation, the most important element protecting the CNS from damage mediated by the immune system was the presence of an intact BBB – hence, the perception of the BBB as a tight and impermeable barrier has also shifted (41).

The BBB is a specialized system of brain microvascular endothelial cells (BMVEC) that are in contact with the endfeet of astrocytes and pericytes and forms a diffusion barrier for hydrophilic blood molecules (35). It ensures constant supply of nutrients for brain cells and controls entry of immune cells into the CNS, which is rare under physiological conditions as the transport across the BBB is strictly limited through both metabolic (enzymes, diverse transport systems) and physical barriers (tight junctions). The enzymatic barrier at the cerebral endothelium can metabolize drugs, nutrients and neuroactive bloodborne solutes and makes the BBB a working and nonstagnant membrane that maintains brain homeostasis. A tight junction is composed of the integral transmembrane proteins occludin, the claudins and the junctional adhesion molecules. These tight junction proteins are connected to the actin cytoskeleton by anchoring proteins such as zonula occludens (ZO)-1, ZO-2 and ZO-3. The presence of tight junctions in the intact BBB has long been assumed to prevent leukocyte migration from the blood into the CNS (42). However, during a variety of pathological conditions of the CNS such as viral or bacterial infections, or during inflammatory diseases like MS or EAE, immunocompetent cells easily cross the BBB (43). Impairment of the BBB during CNS inflammation is thought to result from disruption of junction complexes between BMVEC with subsequent formation of a paracellular route, facilitating entry of leukocytes into the brain parenchyma (44). However, the cause/effect

relationship between BBB disruption and subsequent CNS infiltration of autoreactive leukocytes is still uncertain – solely breaking the BBB does not automatically result in autoimmunity. The undermined immune privilege in this situation might have additional reasons like the appearance of DCs, the establishment of tertiary lymphoid tissue in the meninges, or local immunostimulatory effects of cytokines and chemokines (45).

T cells and cytokines in autoimmunity

The T_H1-T_H2 paradigm

In 1986, Mossman and Coffman proposed the T_H1-T_H2 hypothesis, which classifies T_H cells in different categories depending on their cytokine expression profile and function (46). As described above, T_H1 cells produce IFN-γ, while T_H2 cells secrete IL-4, IL-5, IL-10 and IL-13. These reciprocally inhibitory cytokine profiles are mediated by lineage-specific transcription mechanisms. IL-4 drives the differentiation of T_H2 cells, mediated by signal transducer and activator of transcription (STAT) 6 dependent upregulation of GATA-binding protein 3 (GATA-3), a transcription factor necessary and sufficient for T_H cells to differentiate into a T_H2 phenotype. The transcription factor crucial for T_H1 cell development is T-bet, which amplifies the IFN-γ expression and suppresses the production of T_H2 cytokines. An important signaling molecule leading to this is STAT4, which is activated following IL-12 receptor engagement.

The two T cell subtypes exert distinct functions in adaptive immunity: T_H1 cells mediate cellular immunity, while T_H2 cells regulate humoral immunity and allergic responses (47). However, if these effector functions are uncontrolled and persistent, the onset of autoimmunity, allergy or atopy can be driven. Evidence from clinical observations and from studies on experimental animals supports the idea that uncontrolled T_H2 cell responses cause the development of atopic diseases, such as asthma (48). On the other hand, abnormal T_H1 cell responses have been associated with many autoimmune diseases, including psoriasis, inflammatory bowel disease (IBD), MS and EAE (49,50). Due to the crucial role of IL-12 in T_H1 differentiation, this cytokine and its role in autoimmunity has been extensively investigated.

Interleukin-12: a central cytokine in immunity

The cytokine IL-12 was first identified by Kobayashi *et al.* in 1989 (51) and was shown to be a heterodimeric cytokine of 70kDa consisting of two disulfide linked subunits, a heavy p40 and a light p35 chain (52-54) (**Fig. 2**). The genes encoding p35 and p40 are located

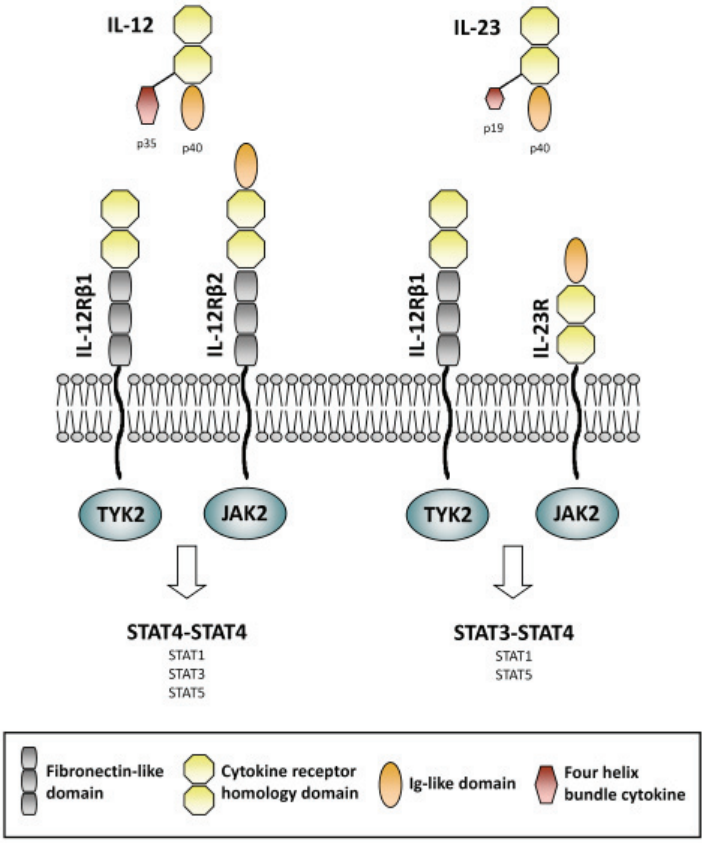

Figure 2: Structure, receptors and main signalling pathways of IL-12 and IL-23.
The heterodimeric cytokines IL-12 and IL-23 – both composed of two disulfide linked subunits – are produced by antigen presenting cells like DCs and macrophages. Although they share similarities in structure, they act on different cells of the immune system. The receptors of IL-12 and IL-23 are both composed of two chains which have to be coexpressed in order to generate functional cytokine binding sites. Following ligand binding, the signal transduction leads to activation of the JAK/STAT pathway. IL-12 predominantly induces homodimerization of STAT4, whereas IL-23 mainly induces formation of STAT3/STAT4 heterodimers.
IL: interleukin, STAT: signal transducers and activators of transcription, TYK: tyrosin kinase, JAK: janus kinase

on different chromosomes and therefore, protein expression is regulated independently of each subunit. p35 is expressed ubiquitously and constitutively at low levels (30) and requires coexpression of p40 for secretion of the biologically active cytokine from the cell. Expression of p40 is highly inducible by microbial products and limited to APCs like monocytes, macrophages, DCs and to a lesser extent B-cells (51,55).

The IL-12 receptor (IL-12R) complex is, just like its ligand, composed of two subunits – β1 and β2 (**Fig. 2**) and is expressed predominantly on NK cells and on activated, but not on resting T cells (56). DCs were also shown to express IL-12R and because these APCs secrete active IL-12 as well, an autocrine effect of this cytokine can be concluded (57). Coinciding with the dominant role of IL-12 in T_H1 polarization, the expression of IL-12Rβ2 on T_H1 cells is tightly regulated, as its expression is maintained by IFN-γ which induces the transcription factor T-bet, whereas IL-4 inhibits the expression of β2. Therefore, expression of a complete IL-12R has been suggested as a determinant of the T_H1 phenotype (58). A variety of different pathogens induce high levels of IL-12p40 and IL-12p70 production in infected hosts, including gram-positive and gram-negative bacteria, parasites, viruses and fungi. IL-12 production is strongly induced in cells of the innate immune system via TLR signaling by microbial products (59). T cell dependent activation of IL-12 production

through the engagement of CD40 on APCs with its cognate ligand CD40L on T cells was also described (60).

The biological effects of IL-12 on its primary targets, $CD4^+$ and $CD8^+$ T cells and NK cells, are manifold. IL-12 induces the production of other cytokines like GM-CSF, TNF, IL-2, but most importantly IFN-γ, which coordinate the ensuing immune response. IFN-γ stimulates the bactericidal activity of phagocytes and enhances the innate immune response. Moreover, it potently induces further IL-12 production by monocytes and PMN. Another important function of the cytokine is the increase of $CD8^+$ T cell and NK cell cytotoxicity which is at least partly achieved by an upregulated expression of adhesion molecules as well as perforin, granzyme B and other granule-associated proteins (61).

IL-12 is in fact best known for its ability to regulate adaptive immunity by directing the polarization of naïve $CD4^+$ lymphocytes towards a T_H1 phenotype and is the most critical cytokine promoting T_H1 differentiation and expansion (62,63). T_H1 cells produce IFN-γ and are crucial for the cell mediated immune response against intracellular pathogens, viruses and bacteria. On the other side, IL-12 and IFN-γ antagonize T_H2 development and therefore limit the production of IL-4, IL-5 and IL-13.

IL-12 in autoimmunity

Due to the central function of IL-12 in inflammation, its properties have been extensively studied in the context of autoimmunity. A number of experimental observations led to the assumption that IL-12 is a key player in MS pathogenesis. Increased levels of IL-12 in serum (64), cerebrospinal fluid (CSF) (65) and MS lesions as well as IL-12 producing peripheral blood mononuclear cells (PBMC) in MS patients could be detected when compared to controls (66). Van Boxel *et al.* showed that the level of IL-12p40 mRNA was elevated in MS patients during the development of active lesions and that in relapsing-remitting MS the increase occurred immediately prior to relapses (67). Ozenci *et al.* could show, that there is an increased number of IL-12Rβ1 and β2 expressing T cells in the CSF of MS patients compared to controls (68). Through various studies in EAE, in which IL-12p40-deficient mice were utilized, recombinant IL-12 administered or the cytokine blocked with anti IL-12p40 antibody, IL-12 had been classified as a disease promoting cytokine. In actively induced EAE in Lewis rats, IL-12 mRNA expression in the CNS emerges early during the initial period and culminates at the peak of disease, consistent with a disease promoting role of this cytokine (69-71). A correlation between the expression of IL-12p40 mRNA and the clinical course of EAE could also be observed in SJL/J mice. Experiments by Segal and colleagues demonstrated impressively the importance of IL-12p40 in EAE: They found, that injection of neutralizing antibodies against IL-12p40 during the priming of donor mice rendered encephalitogenic myelin-reactive T cells harmless (72). Furthermore, it was

reported that anti-IL-12p40 antibody suppresses EAE in adoptive transfer recipients (73). Using IL-12p40-deficient mice, it could further be shown that these mice are completely resistant to EAE induced by active immunization with myelin antigens (72).
In summary, IL-12 appears to have a crucial and non-redundant capacity to polarize T_H1 immunity and to be absolutely vital for the development of autoimmunity. However, as discussed below, it remains to be seen as to whether the properties associated to IL-12 *in vivo* are indeed specific to IL-12 or whether other factors are contributing to the phenomena described above.

Discordant behaviour of IL-12p40- and p35-deficient mice

The fact that T_H1 immunity is considered to be the driving force behind autoimmune diseases such as MS and RA implicates the strong role of IL-12 as well as T_H1 cytokines such as IFN-γ and TNF-α. Surprisingly, mice lacking either IFN-γ or TNF-α were shown to be highly susceptible to autoimmune diseases like EAE (74-76). However, the dogma that T_H1 immunity is a prerequisite for autoimmunity persisted, mainly due to the data obtained from IL-12p40-deficient mice. As discussed above, IL-12p70 consists of two distinctly regulated subunits, p40 as well as p35. The group of M. Gately has generated mice both deficient in IL-12p35 and p40 (77). Initial reports suggested that their behaviour in response to parasitic and mycobacterial infections is equally impeded by the loss of either subunit (77). In EAE however, Becher *et al.* observed a discordant clinical development in p35- and p40-deficient mice. Even though IL-12p40-deficient mice are EAE resistant, p35-deficient mice develop EAE with increased severity compared to wild type (wt) mice (78). This finding was also confirmed by others (79). Conversely, mice lacking the IL-12Rβ1 subunit were EAE resistant, but the β2 subunit was shown to be irrelevant (80). This led to the notion, that p40 may either act as a monomeric cytokine or as a homodimer. Indeed, p40 can also form a homodimer p80 that binds to IL-12Rβ1 and therefore antagonizes IL-12 signalling (81,82). It might also have a direct agonistic function as a macrophage chemoattractant *in vitro* and *in vivo*, but the responsible receptor has not been identified yet (83-85). However, p40 has also been shown to be a subunit of IL-23 and the biology of this newly identified cytokine will be described in the following.

Interleukin 23: changing the verdict on IL-12 function in inflammation and autoimmunity

The recently described cytokine IL-23 is also a member of the IL-6 cytokine-family and has similar but discrete functions from IL-12 (86). Like IL-12, it is comprised of two disulfide-linked subunits designated p40, which is shared by IL-12, and a unique protein termed p19

(**Fig. 2**). The p19 component is produced in large amounts in activated macrophages, DCs, T cells and endothelial cells where its production is directly induced by CD40-crosslinking and indirectly by IL-1 and IFN-β (87,88). T_H1 cells express more p19 mRNA than do T_H2 cells, however, among these cell types, only activated APCs such as monocytes, macrophages and DCs concomitantly express IL-12p40 (89,90).

The IL-23 receptor consists of the IL-12Rβ1 subunit which is shared with the IL-12 receptor and the IL-23R subunit (91) (**Fig. 2**). IL-23R subunit expression has been demonstrated on activated/memory T cells, NK cell lines, EBV-transformed B-cells, but also on monocytes, macrophages and DCs (91,92). IL-23 stimulation leads to activation and phosphorylation of different STATs, joining IL-12 and type I IFNs by also using STAT4 as a transcription factor. However, in contrast to IL-12, IL-23 preferentially activates STAT3, with its receptor binding to STAT4 being quite weak compared to that of IL-12R (91), a fact that might explain why the biological effects of these cytokines are similar but not identical (**Fig. 2**). In addition to the presence of IL-23R on accessory cells, the expression of the IL-23R chain was found to be much higher on $CD4^+CD45RB^{lo}$ memory cells than on $CD4^+CD45RB^{hi}$ naïve cells (91). The opposite pattern of expression was observed for IL-12Rβ2. This unique expression pattern of IL-23R is consistent with a stronger functional response of memory T cells to IL-23 when compared to naïve T cells (86,93-95).

IL-23 in autoimmunity: making sense of the observed discrepancies

Over the past ten years, it was believed that the ability of IL-12 to strongly promote the development of T_H1 cells made it an ideal target for the treatment of T_H1 cell-mediated diseases like autoimmunity. The role of endogenous IL-12 had been addressed by using IL-12p40-deficient mice or by administering anti-IL-12p40 antibodies. However, although the inflammatory response in these models was characterized by IFN-γ production, the role of IFN-γ in the development of inflammation was less clear. In two different disease models of autoimmunity, EAE and CIA, it could be shown that, while p40 was important for the development of autoimmune inflammation, the absence of IFN-γ resulted in the development of a more severe pathology (74,76,96). The discrepancy between the role of IL-12p40 and IFN-γ in these models of inflammation was inconsistent with a linear model in which IL-12 drives a pathological autoimmune T_H1-cell response and indicated an important role of IL-12 in the development auf autoimmunity.

But with the controversial observed phenotypes of mice deficient in IL-12p40 or IL-12Rβ1 compared to those lacking either IL-12p35 or IL-12Rβ2, it became clear that, while p40 is absolutely critical for the development of different experimental diseases, IL-12 is completely dispensable (78-80,97,98). The discovery of IL-23, which shares the p40 chain and the IL-12Rβ1 with IL-12, has started to provide an explanation to the numerous

observations existing in the literature and provided indirect evidence that IL-23 has an important function in controlling certain infections and T cell mediated autoimmune responses.

The first indirect evidence for the importance of a p40-containing cytokine other than IL-12 came from studies using p35 gene-targeted mice in EAE. $p35^{-/-}$ mice lack bioactive IL-12 but have normal levels of IL-23 and offer a unique tool to differentiate the role of IL-12 from IL-23. While EAE could not be induced in IL-12p40-deficient mice, the disease was even more severe in p35- or IL-12Rβ2-deficient mice than in wt mice (78-80) suggesting a redundancy of the IL-12 system in the induction of EAE. It could be shown that in the susceptible IL-12p35-deficient mice the disease was associated either with a downregulation of the autoantigen-specific T_H1 response (78) or even a deviation to T_H2 (79). This confirmed the importance of biologically active IL-12 in orchestrating T_H1 type immune responses, but illustrated that EAE can develop under T_H2 conditions. These findings together with the resistance of p40-deficient mice suggested that other p40-containing cytokines, such as IL-23, are required for establishment of the disease. In experiments in which a bone marrow chimeric mouse model was used so that p40 was deleted from the CNS parenchyma, but not the systemic immune compartment, it could be shown that p40 is not absolutely required for the infiltration of inflammatory cells into the CNS but that p40 produced by cells resident to the CNS is essential for the maintenance of encephalitogenicity of T cells migrating into the CNS (78). This finding was confirmed in IL-23p19-deficient mice, which are, similar to p40 knockout mice, completely resistant to EAE induction (99). Cua *et al.* further demonstrated that administration of IL-23 into the CNS of p19 knockout mice just before disease onset can abrogate the resistance to EAE whereas injection of IL-12 into IL-12p40-deficient mice had no effect. However, when the IL-12 treatment (at an early time point in the disease) was followed by administration of IL-23 into the CNS, EAE comparable with that seen in wt mice was induced.

A very similar role for IL-12 and IL-23 has been observed in the induction of another autoimmune disease, CIA. Mice specifically lacking IL-23 (p19-deficient) and those animals lacking both IL-12 and IL-23 (p40-deficient) were resistant to CIA induction. Neither p40- nor p19-deficient mice revealed pathology upon histological examination of joints whereas mice specifically lacking only IL-12 (p35-deficient) were highly susceptible to CIA. Disease severity appeared to be more intense in p35-deficient mice compared with wt mice as was also seen in EAE experiments. Disease recovery was delayed in mice lacking only IL-12. Thus, IL-23, but not IL-12, is necessary for the development of joint autoimmune inflammation and the absence of IL-12 may result in a more severe disease. It is now established that IL-23 drives the development of $CD4^+$ T cells into a new subset of IL-17 producing T_H cells which can be linked to autoimmune diseases (14,15). Thus, the

development of a distinct lineage of $CD4^+$ T cells expressing IL-17 now further explains the different actions of IL-12 and IL-23 in regulation of tissue inflammatory reactions and autoimmunity.

T_H17 cells

The first time, a unique IL-17A producing T_H cell subset different from the classic T_H1 and T_H2 paradigm has been suggested was in 2000 (100). Infante-Duarte and colleagues observed that naïve T cells primed by a lysate of B. burgdorfei developed a phenotype distinct from T cells primed under T_H1 and T_H2 conditions, with much higher IL-17A secretion. They also proposed that IL-6 might play a role in the development of these IL-17A-producing T_H cells. Gurney and colleges could then show that IL-23 enhances IL-17 production from memory but not naïve $CD4^+$ T cells (97). Together, these observations led to the hypothesis that IL-23 is required for the development and/or expansion of the now – based on the expression of their characteristic cytokine – T_H17 termed new lineage of inflammatory T_H cells (14,15,101). The characteristic cytokine expression profile of T_H17 cells includes IL-17A and IL-17F, IL-22, IL-21 and TNF-α (102).

The transcription factor RORγt, a retinoid orphan nuclear receptor, is specifically expressed in both human and mouse T_H17 cells and necessary and sufficient to induce IL-17A, IL-17F and IL-23R expression (103). RORγt upregulation is dependent on STAT3, but for maximal IL-17 production functional STAT4 also seems to be required (104,105). Recently, Yang *et al.* reported, that RORα is also expressed by T_H17 cells, but compared to RORγt it seems to be a minor player in T_H17 differentiation (106).

It is still a matter of debate, which factors and cytokines are responsible for the initial regulation of T_H17 cell lineage commitment. The first two papers describing the *de novo* induction of T_H17 cells reported that IL-23 was, in the absence of IFN-γ and IL-4, the critical T_H17 inducing factor (14,15). However, shortly afterwards it was demonstrated that TGF-β and IL-6 were sufficient for T_H17 differentiation and also induced RORγt upregulation (107-109). These findings together with the IL-23R being absent on naïve T cells argue against IL-23 being required for initial T_H17 differentiation and raise the question of the role of IL-23 for T_H17 cells. The most common hypothesis is that IL-23 is important for the expansion of differentiated T_H17 cells or maintainance of IL-17 production. These assumptions stem from *in vitro* observations that the presence of IL-23 during culture of activated cells results in increased proliferation of activated T cells (93) and increased frequencies of IL-17 secreting cells (101) and that IL-23 was required during restimulation of TGF-β plus IL-6-stimulated cells to maintain their IL-17 production (109). It has also been proposed, that IL-23 might stabilize the T_H17 phenotype through STAT3-dependent mechanisms (106,110) or that IL-23 is a survival factor for T_H17 cells (111).

The fact that TGF-β is involved in T_H17 induction was unexpected, since TGF-β is known to induce the expression of FoxP3 and the development of T_{reg} cells. Therefore, IL-6 seems to influence the action of TGF-β by inhibiting T_{reg} cells on one side (112) and by upregulating T_H17 promoting factors such as IL-23R and IL-21 in T cells on the other (103,110). The discovery of a crucial regulatory role for retinoic acid in promoting FoxP3 induction and inhibiting T_H17 cell differentiation further supports a reciprocal relationship between inducible T_{reg} and T_H17 cells (113). IL-21 is secreted by T_H17 cells and acts in an autocrine manner to further promote T_H17 cells. It might, however, not be essential for T_H17 lineage commitment or their pathogenic functions. Furthermore, IL-1β was found to promote T_H17 cell development or proliferation in the presence of IL-6 and TGF-β (109).

Like T_H1 and T_H2 cytokines reciprocally inhibit the other cell type, T_H17 cells are also negatively regulated by several cytokines. On the one hand by T_H1 and T_H2 cytokines like IL-12, IFN-γ, or IL-4, which addition to cultures inhibits IL-23- as well as TGF-β plus IL-6-stimulated differentiation of mouse and human T_H17 cells (14,15,114) and on the other hand, T_H17 cells are inhibited by IL-10 and IL-27. IL-10 has been proposed to be a self-regulatory mechanism as this cytokine is expressed by T_H17 after continued exposure of differentiating T_H17 cells to TGF-β and IL-6 (115). IL-27 was first identified as a T_H1 inducing, proinflammatory cytokine. However, it has recently been demonstrated that it exerts immunoregulatory functions by suppressing the development and proliferation of T_H17 cells, thereby limiting autoimmune inflammation (116,117).

The factors inducing T_H17 differentiation in humans are less clear but seem to differ from those required in mice to some extent. However, the phenotypes of human and murine T_H17 cells comprise many similarities. In both cases, IL-17A, IL-17F and IL-22 are hallmark cytokines and the expression of the IL-23R and the chemokine receptor CCR6 additionally define this subset (113).

Most importantly, T_H17 cells are now closely associated with a number of murine and human autoimmune disorders like CIA, IBD, MS and EAE. The role and function of the T_H17 cytokines IL-17A, IL-17F and IL-22 will be described in the following.

IL-17A and IL-17F

IL-17A is the founding member of the IL-17 family of cytokines, which includes five other members, named IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F. IL-17A was originally cloned and described by Rouvier *et al.* (118). IL-17F shares the greatest homology (55%) with IL-17A and the genes encoding these two cytokines are located in close proximity on the same chromosome in mice and humans. IL-17A and IL-17F are produced by T_H17 cells, whereas IL-17B, IL-17C and IL-17D are produced by non T cell sources. IL-17A and IL-17F can either exist as IL-17A homodimers and IL-17F

homodimers or as IL-17A-IL-17F heterodimers (119). The IL-17 receptor family consists of five, broadly distributed receptors with individual ligand specificities. Within this family of receptors, IL-17R (also known as IL-17RA) is the best characterized, it binds IL-17A and is expressed in multiple tissues: vascular endothelial cells, peripheral T cells, B-cell lineages, fibroblast, lung, myelomonocytic cells and stromal cells. Recently, IL-17RA was shown to also form a heterodimer with IL-17RC, (120) another member of the IL-17R family and there is evidence that IL-17RC binds IL-17F (121). IL-17A also binds to IL-17RC, but altogether it is still not clear whether IL-17 and IL-17F homodimers, as well as the IL-17–IL-17F heterodimer, preferentially interact with IL-17RA and IL-17RC homodimers or heterodimers. Upon engagement, activation of nuclear factor-κB (NF-κB) and JUN amino-terminal kinase (JNK) signaling pathways are induced in a tumor-necrosis factor receptor (TNFR)-associated factor 6 (TRAF6)-dependent manner (122). ACT1, another adaptor protein has been identified recently. It serves as an immediate and essential signaling component of IL-17R (123), associates with the intracellular region of IL-17RA and is required for NF-κB activation and the induction of many pro-inflammatory genes by IL-17. Both IL-17A and IL-17F mainly act on fibroblasts, epithelial and endothelial cells, exert pro-inflammatory effects and induce cytokines (IL-6, GM-CSF, G-CSF), chemokines (IL-8, CXCL1, CXCL10) and matrix metalloproteinases (16). IL-17A and IL-17F are also key cytokines for the recruitment, activation and migration of neutrophils. Furthermore, they have also been reported to be important in host defense against bacterial infections in mice.

IL-17A and F in autoimmunity

Already in 1998, Teunissen and colleagues described the upregulation of IL-17A in psoriatic skin (124) and since then potential roles of this cytokine in other autoimmune diseases like CIA, IBD, MS and EAE have been suggested. For example, abrogation of IL-17A prior to disease onset mildens antigen-induced arthritis in mice and treatment with neutralizing antibodies after disease onset decreases joint damage (125,126). Recently, IL-17 has also been demonstrated to regulate auto-antibody production (127). IL-17A deficient mice or mice treated with an IL-17R antagonist develop reduced adjuvant-induced arthritis (125,128) but are susceptible to EAE, albeit with a delayed onset and reduced severity. Even though more and more data indicate that IL-17 cannot be the most important cytokine in EAE or MS, many reports suggesting IL-17 to be a key player in these diseases have accumulated during the recent years. IL-17A was described to be upregulated in CNS lesions of patients with MS (129), neutralization of IL-17 reduces the severity of EAE (99) and, as mentioned above, IL-17A-deficient mice show delayed onset and reduced maximum-severity scores in EAE (130). Furthermore, EAE could be induced after transfer

of *in vitro* polarized T_H17 cells (101).
Taken together, IL-17 certainly seems to play a pathological role in many autoimmune diseases, although it is very unlikely to be the only effector cytokine in autoimmunity. Efficacy, safety and tolerability studies with a monoclonal anti-IL-17 antibody (AIN457) are currently carried out in Crohn's disease (CD) and psoriasis that are resistant to current therapies and the results of these clinical trials will clearly give new insights in the contributions of IL-17 to these diseases (102).

IL-22

IL-22 was first described in 2000 as an IL-9 induced gene and termed "IL-10 related T cell-derived inducible factor" (IL-TIF). Together with IL-19, IL-20, IL-24 and IL-26 it belongs to a family of cytokines structurally related to IL-10 (131). The IL-22 gene was mapped near the IFN-γ gene on chromosome 10 in mice and on chromosome 12 in humans. Unlike in humans or the genomes of BALB/c and DBA/2 mice, genomes of other mouse strains such as C57Bl/6, FVB and 129 possess two IL-22 genes, designated IL-22 α and β. The murine IL-22β gene contains several single nucleotide changes and a 658 nucleotide deletion covering the first noncoding exon and a segment of a putative promoter, suggesting that the IL-22 β gene may not be expressed (132).
IL-22 signals through a class II cytokine receptor complex composed of two chains - IL-22R1 and IL-10R2. IL-22R1 is also utilized by IL-20 and IL-24 and IL-10R2 is shared with IL-10, IL-28 and IL-29. The expression of IL-22R1 could only be detected in a limited number of tissues such as skin, liver, lung, kidney and pancreas, whereas cells of the immune system do not express IL-22R1 even under stimulated conditions (133). Concordantly, IL-22 failed to stimulate immune cell functions such as cytokine secretion and surface marker expression but elicited very strong responses from many epithelial cells or cell lines, including acinar cells, hepatocytes, keratinocytes and colon epithelial cells (131,134,135). In addition to the cell surface IL-22 receptor complex, a soluble single chain IL-22 receptor exists, termed IL-22 binding protein (IL-22BP), which antagonizes IL-22 cellular binding and signaling *in vitro* (136). The signaling of IL-22 following binding to its receptor includes activation of JAK1 and Tyk2 tyrosine kinases, which leads to STAT3- and, to a lesser extent, to STAT1- and STAT5-phosphorylation. IL-22 was also found to activate the ERK, JNK and p38 MAPK in the rat hepatoma cell line H4IIE.
Wolk *et al.* have shown that in peripheral blood mononuclear cells (PBMCs), the major source of IL-22 are activated (memory) T cells, especially upon T_H1 polarization and NK cells (137,138).
However, soon after the characterization of T_H17 cells it became clear, that this T cell subtype is the dominant IL-22 producer, as demonstrated at both the mRNA and protein

levels and now it is clearly established that IL-22 is another effector cytokine produced by T_H17 cells (139). Additionally, IL-22 secretion in monocytes and $CD11c^+$ DCs as well as $CD8^+$ T cells and γ/δ T cells after IL-23 stimulation has been reported (140,141). IL-22 drives the production of many antimicrobial peptides, including β-defensins, S100-family proteins and regenerating-gene (Reg)-family proteins and protects against extracellular bacterial pulmonary and enteric infections (142-144).

IL-22 in autoimmunity

The first evidence of a role of IL-22 in autoimmunity stems from observations made in psoriasis, as IL-22 is upregulated in psoriatic skin (145) and induces many psoriatic features in cultured reconstituted human epidermis *in vitro*. (146). After injection of IL-23 into the ear, an inflammatory skin phenotype with IL-22 and IL-17 expressing T_H17 cell infiltration and epidermal acanthosis is induced (140) and diminished in IL-22-deficient mice. Furthermore, the inflammatory skin disease in a murine model of psoriasis is ameliorated after IL-22 antibody treatment (147).

An upregulation of IL-22 has also been described in autoimmune diseases like CD and ulcerative colitis (UC), as well as in preclinical mouse IBD models or RA patients (135,148).

In addition to its proinflammatory role, protective functions of IL-22 have also been described. For example, IL-22 protects the liver injury by enhancing the growth and survival of hepatocytes (149). Studies with both IL-22- and IL-17-deficient mice further supported a protective function of IL-22 (150) and IL-22 has been shown to be protective in a rat model of experimental autoimmune myocarditis (151). In conclusion, both pathogenic and protective functions of IL-22 in autoimmune diseases have been observed, depending on the specific situations and target cells.

IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation

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Abstract

The clear association of the T_H17 phenotype with autoimmune pathogenicity implicates T_H17 cytokines as critical mediators of chronic autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE). To study the actual impact of IL-17 on CNS inflammation, we generated transgenic mice in which the expression of high levels of IL-17A can be initiated by cre-recombination. While the general overexpression of IL-17A leads to skin inflammation and granulocytosis, T cell driven IL-17A expression does not impact visibly on the development and health of the mouse. In the context of EAE, here we demonstrate that neither the T cell-driven overexpression of IL-17A nor its complete loss have a significant impact on the development of clinical disease. Since T_H17 cells also produce IL-17F, which evidently acts through the same receptor complex as IL-17A, it is feasible that it could compensate for the loss of IL17A in IL-17A^{null} mice. To determine the actual function of IL-17F in autoimmune inflammation of the CNS, we next generated IL-17F^{null} mice. They not only lack IL-17F, but also produce significantly less IL-17A. Nevertheless, they too are fully EAE susceptible and display unaltered emergence, expansion and polarization of auto-reactive T cells during EAE. We conclude that IL-17A and IL-17F, while prominently expressed by encephalitogenic T cells, do not or only marginally contribute to the development of autoimmune CNS-disease.

Introduction

Multiple sclerosis (MS) and its animal model EAE are characterized by the invasion of self-reactive T_H cells into the CNS, leading to demyelination, axonal loss and neurological impairment (152). Upon activation, T_H cells can differentiate into different effector cells, depending on the makeup of the priming immune synapse as well as the cytokines present (153). For over a decade, IFN-γ secreting T_H1 cells were thought to be the pathogenic population central to the pathogenesis of autoimmunity, reasoned by the clear association of the T_H1 effector type to diseases like rheumatoid arthritis, multiple sclerosis and type I diabetes. In their respective animal models, however, the loss of the major T_H1 cytokines, IFN-γ, IL-12 and IL-18 surprisingly did not hamper disease development (76,78,154). In fact, IFN-γ and IL-12 deficiency lead to clinically even more severe inflammation in CIA and EAE (76,78,96). With these discoveries the simplistic notion that T_H1 cells and their respective cytokines are the culprits of autoimmunity had to be revised. In contrast to IL-12, its relative IL-23 was found to be essential for the development of EAE and CIA (99). The finding that IL-23 induces the expression of IL-17A by T_H cells then gave rise to the notion that not T_H1, but IL-17A secreting T_H cells (T_H17) are the main pathogenic population in autoimmune diseases (155). The respective hypothesis was strongly supported by adoptive transfer models, in which the transfer of IL-17A producing effector cells into wild type hosts resulted in the initiation of autoimmunity (156). The *de novo* lineage commitment of naïve T cells towards IL-17A secretion is dependent on TGF-βR engagement whereas IL-6 has been identified as a differential co-stimulus directing cell fate towards $T_H17$committment (109). IL-17A secretion is considered the hallmark of T_H17 function, as it exhibits strong pro-inflammatory properties (157) and is widely held as being the major driving force in the pathogenesis of autoimmunity. Despite the plethora of data published on IL-17A, implicating its function in physiological processes, hardly any studies reveal a true causative association. Here, we have generated mice in which specifically T cells overexpress IL-17A regardless of their state of activation and found that even massively increased levels of IL-17A producing cells in the inflamed CNS has no impact on the pathogenesis of EAE. Also, we confirmed that IL-17A$^{-/-}$ mice are fully susceptible to EAE. It is feasible that other T_H17 cytokines can compensate for the loss of IL-17A. The closest associate to IL-17A is IL-17F and (158,159) is encoded syntenically, shares around 50% sequence homology and a virtually identical pattern of expression with IL-17A (160). IL-17A/F heterodimers were described (161) and shown to signal through the same receptor complex (120). In order to determine whether IL-17F contributes to the pathogenicity of T_H17 cells, we generated IL-17F^{null} mice and explored their susceptibility to EAE. We found that in addition to lacking IL-17F, they express lower levels of IL-17A.

Surprisingly however, we discovered IL-17F$^{-/-}$ mice also to be fully susceptible to EAE, indicating that, while T_H17 cells associate with an encephalitogenic phenotype, neither IL-17A nor IL-17F appear to contribute to the pathogenic function of T_H17 cells *in vivo*.

Results

Transgenic targeting of IL-17A to T cells does not alter the pathogenesis and clinical development of MOG-induced EAE.

To address the impact of IL-17A expressed by CNS invading T cells on the pathogenesis of EAE, we generated a mouse conditionally overexpressing IL-17A and EGFP after excision of a loxP-flanked transcriptional STOP-cassette (termed IL-17A^{ind}) (**Supplemental Fig.1**). In order to verify the function of the transgene, IL-17A^{ind} were crossed with the *cre-deleter* strain. These mice show early signs of skin inflammation and ultimately a developmental retardation clearly visible from P4-6 on, as can be seen in Supplemental Figure 2 A. On the cellular level the IL-17A overexpression (**Suppl. Fig. 2 B**) leads to a massive increase in the number of granulocytes throughout the body, especially in bone marrow, spleen and blood (**Suppl. Fig. 2 C-D**). Chemical analysis of the blood compartment revealed an anaemia-like phenotype which can be expected to accompany granulocytosis (**Suppl. Fig. 2 E**). We are in the process of further elucidating the clinical impact of this genotype (manuscript in preparation). Crossing the IL-17A^{ind} to CD4-Cre expressing mice (CD4-IL17A^{ind}) generated a T cell repertoire in which only T cells express IL-17A and EGFP (**Supplemental Fig.1 D and Fig.1 A**). Expression of IL-17A by T cells in CD4-IL17A$^{ind/+}$ mice did not result in an overt phenotype neither in the macroscopic constitution of the mice nor in the cellular compositions of thymus, spleen and lymph nodes (**Supplemental Fig.1 D** and data not shown). To verify the increased IL-17A secretion we performed IL-17A-specific ELISA assays with FACS-sorted CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ CD4$^+$ T cells 48 h after stimulation by CD3 and CD28 cross-linking. As

	Day of onset[A B]	Incidence	Max. score[A B]
CD4-IL17A$^{ind/+}$	10 ± 0.4	90 % (9 / 10)	2.75 ± 0.37
IL17A$^{ind/+}$	10 ± 0.3	90 % (9 / 10)	2.53 ± 0.17
IL-17A$^{-/-}$	10 ± 0.3	91 % (32 / 35)	2.36 ± 0.13
IL-17A$^{+/+}$	9 ± 0.4	100 % (46 / 46)	2.75 ± 0.19
IL-17F$^{-/-}$	11 ± 0.3	95 % (37 / 39)	2.68 ± 0.10
IL-17F$^{+/-}$	10 ± 0.3	90 % (38 / 42)	2.74 ± 0.12
IL-17F$^{+/+}$	12 ± 0.7	100 % (17 / 17)	2.67 ± 0.15

[A] Average of diseased mice
[B] SEM indicated

Table I: Detailed clinical development of EAE in IL-17A over-expressing, IL-17A and IL-17F deficient mice.

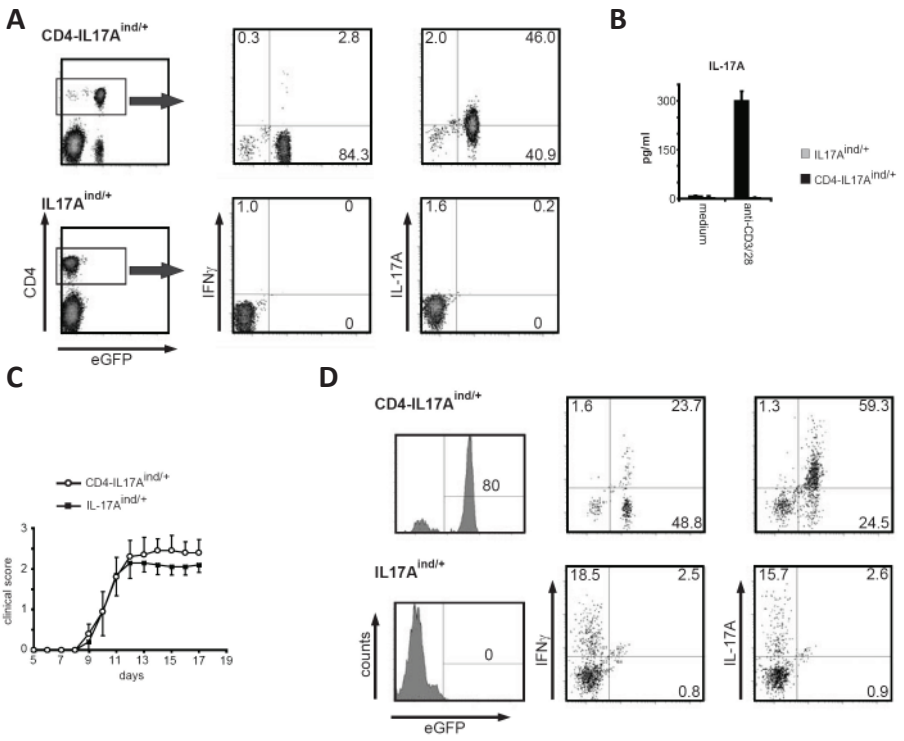

Figure 3: IL-17A overexpression does not exacerbate EAE.
(A) Splenocytes from naive CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ littermates were restimulated in culture and subsequently stained for CD4 and IL-17A. Percentages of EGFP$^+$IL-17A$^+$ or EGFP$^+$IFN-γ$^+$ CD4$^+$ T cells are given in the quadrant corners after gating on CD4$^+$ T cells. Data shows a representative of three independent experiments. (B) 1x10^5 FACS-sorted CD4$^+$ T cells were cultured for 36 h in the presence or absence of anti-CD3 and anti-CD28, after which IL-17A secretion was measured by ELISA. (C) Clinical scores after MOG$_{35-55}$-induced EAE are not significantly altered by increased IL-17A expression in CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ littermates. Error bars represent +/- SEM. Data shown represents one out of two independent experiments. (D) Lymphocytes isolated from the diseased EAE brain and spinal cord at d 14 from CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ littermates were surface stained for CD4 and examined for EGFP-expression. Further staining for CD4, IL-17A and IFN-γ was performed. Percentages of EGFP$^+$IL-17A$^+$ or EGFP$^+$IFN-γ$^+$ are given in the quadrant corners. Plots shown are gated on CD4$^+$ CNS-derived T cells.

expected, CD4-IL17A$^{ind/+}$ CD4 T cells produce and secrete highly elevated levels of IL-17A compared to IL-17A$^{ind/+}$ T cells, where overexpression of the cytokine is not induced (**Fig. 3 B**). Next, we immunized CD4-IL17A$^{ind/+}$ mice with MOG$_{35-55}$/CFA and followed the onset and progression of EAE. Surprisingly, no significant clinical differences were observed between CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ littermates (**Fig. 3 C and Table I**). Consistent with the clinical disease cellular CNS invasion was virtually identical in both groups as measured by flow cytometry (data not shown). From all CNS fractions analyzed no significant alterations were observed with respect to IFN-γ secreting cells (**Fig. 3 D**). However, CNS extracts from CD4-IL17A$^{ind/+}$ mice presented with a clearly identifiable and highly significant increase in IL-17A$^+$ T cells compared to IL-17A$^{ind/+}$, despite indistinguishable clinical scores. Taken together, an increase in T cell derived IL-17A in the inflamed CNS during MOG-induced EAE does not result in an appreciable alteration of the disease course.

IL-17A function is redundant in the development of EAE

In order to improve the understanding of the role of the T_H17 effector type in autoimmunity we next analyzed the impact of loss of IL-17A on the autoreactive T_H17 response in EAE. Despite the close association of IL-17A with the inflammatory milieu in EAE, the loss of IL-17A does not fundamentally impede the induction of the disease, which is similar to the observations made by Iwakura and colleagues (130) (**Fig. 4 A**). In the course of EAE, shown in Fig. 4 A, there is an apparent mild decrease of disease severity. Overall, the IL-17A deficient mice are statistically less severely sick (ANCOVA, P=0.015). However, the difference in the course of the disease between wildtype and IL-17A deficient mice accounts for only 1.6 % of the variance in the experiment, which enumerates how small the effect size is compared to the variance in the system (e.g. the "mouse identity" accounts for 24 % of the variance). In a detailed analysis of the pooled data, we observed only a minimal yet statistically significant (two-tailed t-test, p=0.013) difference in the day of onset of disease and an insignificant decrease in incidence and the maximal severity of disease (**Table I**). The non-essential role of IL-17A in EAE development may be due to the involvement of other T_H17 associated factors. To elucidate the quality of the T_H17 response in the immunized IL-17A deficient mice

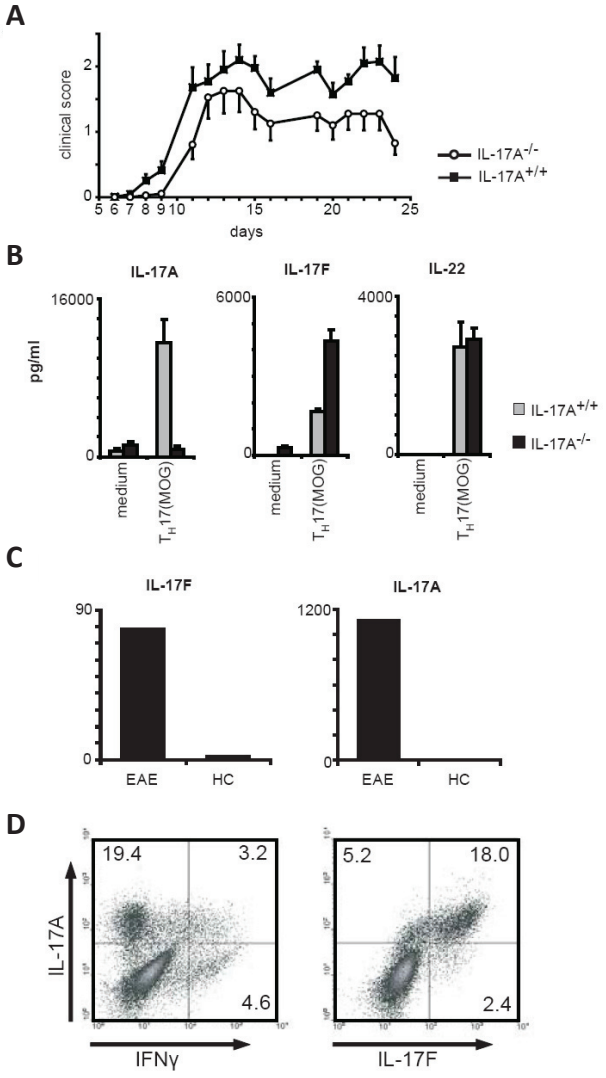

Figure 4: IL-17A is redundant in the induction of EAE which could be due to a compensatory increase of IL-17F production.
(A) EAE was induced in IL-17A$^{-/-}$ and IL-17$^{+/+}$ mice by immunization with MOG_{35-55}/CFA. The graph shows the development of EAE according to clinical scores (n=10, SEM as indicated) in one out of two independent experiments. (B) T_H17-cytokine profile measured by ELISA of splenocytes isolated from mice with active EAE and restimulated with MOG_{35-55} under T_H17 polarizing conditions for 2 days. (C) Comparative mRNA expression analysis of IL-17F and -17A in the cerebellum of mice at peak EAE vs. healthy controls, HC. The data represents one of two independent experiments (n=4). (D) T_H17 cells were generated in vitro from MOG_{35-55}-immunized C57BL/6 mice. Seven dpi splenocytes were harvested, T_H17-polarized and analyzed by intracellular cytokine staining for IL-17A, IL-17F and IFN-γ. Shown is a representative of three independent experiments.

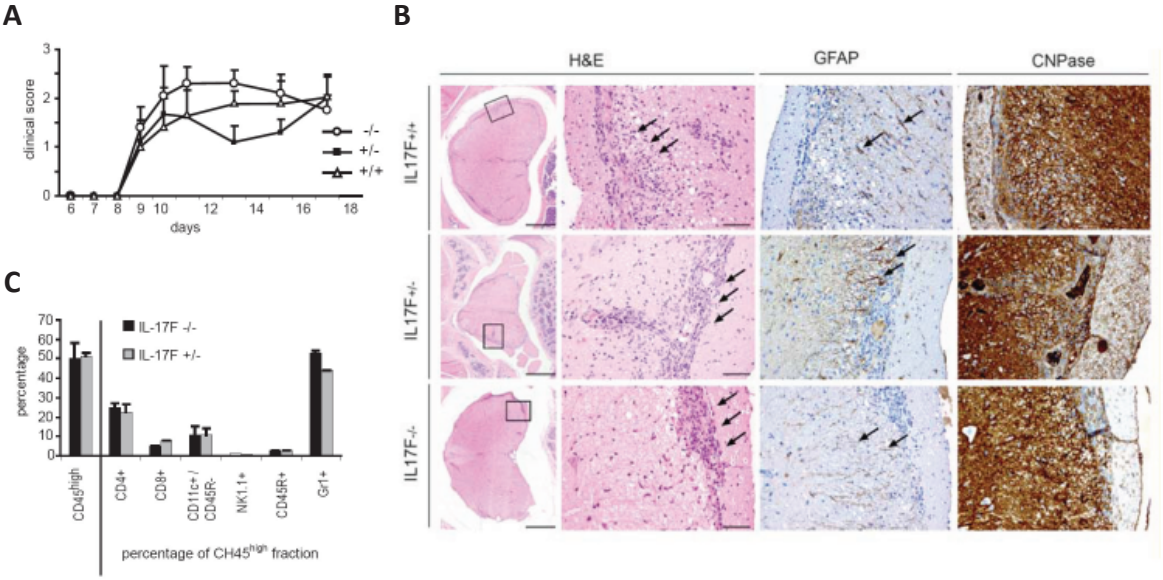

Figure 5: IL-17F is not required for the development of EAE.
(A) EAE was induced in IL-17F$^{-/-}$, $^{-/+}$ and $^{+/+}$ mice by immunization with MOG$_{35-55}$/CFA. The graph shows the development of EAE according to clinical scores in one out of five independent experiments. (B) Spinal cord cross-sections in accordance with clinical EAE scores displayed similar inflammatory lesions (H&E staining, 1st and 2nd column, arrows). Inflammation caused an impairment of myelinated structures (staining for CNPase, 4th column) and induced a reactive astrogliosis (staining for GFAP, 3rd column). All stainings were performed on serial sections. Scale bars: 500 µm for 1st, 50 µm for 2nd, 3rd and 4th column. Insert in 1st column represents the area shown in 2nd, 3rd and 4th column. (C) Detailed analysis of infiltrating lymphocytes into cerebellum and spinal cord was performed by cytofluorometric analysis of surface marker staining. CD45^{high} cells represent the CNS-invading leukocytes which were gated on for detailed analysis.

we analyzed their cytokine secretion upon *in vitro* restimulation with MOG$_{35-55}$ peptide under T$_H$17 polarizing conditions (**Fig. 4 B**) or under the influence of IL-23 alone (data not shown). Surprisingly, while the IL-22 levels were equal in T cells obtained from IL-17A deficient and wild type control mice, IL-17F secretion was found to be consistently and drastically increased in the lymphocytes of IL-17A deficient mice. This specific increase of one T$_H$17 cytokine suggests the possibility of a compensatory role of IL-17F in disease development.

IL-17F is expressed by T$_H$17 cells and is abundant in the inflamed CNS

A screen performed by quantitative RT-PCR of cerebellum of mice with active EAE revealed that IL-17F like IL-17A is highly expressed in the lesioned CNS as compared to cerebellum of healthy controls (**Fig. 4 C**). To assess the source of IL-17F in the context of EAE we restimulated *in vivo* primed splenocytes *in vitro* with MOG$_{35-55}$ under T$_H$17 polarizing conditions and subsequently analyzed the cytokine profile by intracellular cytofluorometric analysis. Figure 4 D shows that IL-17F expression is restricted to MOG-

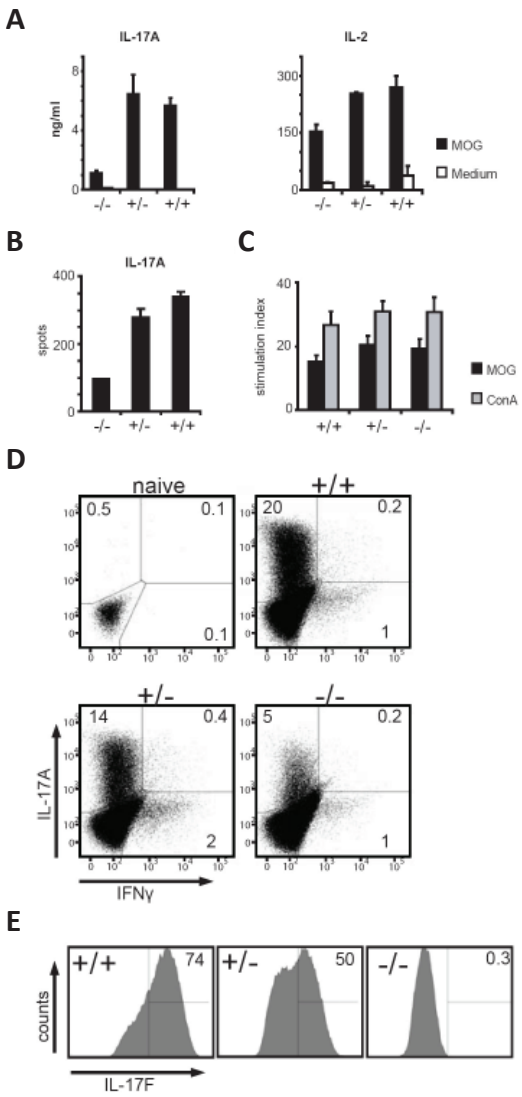

Figure 6: Loss of IL-17F does not impact on T cell proliferation upon T_H cell priming and does not lead to a compensatory upregulation of IL-17A.

(A-C) Mice were immunized with MOG_{35-55}/CFA and lymphocytes isolated from LN prior to disease onset on 7 dpi. Cells were rechallenged with 50µg/ml of MOG_{35-55} and IL-17A and IL-2 was measured by ELISA (A) and ELISPOT (B). (C) Proliferation of effector T_H cells was measured by thymidin incorporation. (A-C) Shown is a representative of at least three independent experiments. Error bars indicate SEM of measured replicates. (D) Splenocytes from naive mice were polarized towards the T_H17 effector type in vitro and IL-17A, IL-17F and IFN-γ was measured by intracellular cytokine staining. Dot plots are gated on T_H cells ($CD4^+$), histograms are gated on T_H17 cells ($CD4^+$, IL-17A$^+$).

responsive and potentially encephalitogenic $CD4^+$ T_H cells that are also secreting IL-17A. The findings that both cytokines are found in the inflamed CNS and mark those highly pathogenic T_H17 cells that have been associated with autoreactive lesions suggests that IL-17F is a encephalitogenic cytokine with functional relevance in CNS autoimmune inflammation.

Generation and analysis of the IL-17F deficient mice

To ultimately determine whether IL-17F contributes to the development of EAE, we generated IL-17F deficient mice by the replacement of exons 2 and 3 with a lacZ reporter cassette (Supplemental Fig. 1). In the following experiments IL-17F deficiency was confirmed by ELISA (Supplemental Fig. 1). The homozygous offspring is viable and shows neither developmental malformation nor any evident immunodeficiency under SPF conditions. There are no apparent alterations in the cellular composition of the immune system in homeostasis as is shown by FACS analysis for spleen and thymus in Supplemental Table 1. Up to 42 mice per group (+/+, +/−, −/−) in 6 independent experiments were immunized with MOG_{35-55}/CFA and the clinical development of EAE was monitored daily. Against our expectations, the lack of IL-17F did not have any visible consequences for the clinical EAE development as shown in Figure 3 A. IL-17F$^{-/-}$ mice did not show any alteration in the day

of disease onset, maximum score or incidence when compared to IL-17F competent (+/+, +/−) mice (**Table I**). In accordance with clinical EAE scores, histological analysis of spinal cord cross-sections displayed no discernable features between wild type mice and hemizygous IL-17F$^{+/-}$ and/or homozygous IL-17F$^{-/-}$ mice. Inflammation caused a severe impairment of myelinated and axonal structures, subsequently inducing reactive astrogliosis (**Fig. 5 B**). Detailed analysis of CNS-infiltrating cells by flow cytometry revealed no change in cell numbers or makeup of CNS invading leukocytes (**Fig. 5 C**).

We did not observe any relevant difference in the capacity of IL-17F$^{-/-}$ mice to initiate CD4 T cell priming and the effector T cell response. IL-17F$^{-/-}$ and control mice were immunized against MOG_{35-55} and primed lymphocytes were isolated from draining LN prior to disease onset (**Fig. 6 A-B**) and were subsequently challenged *in vitro* with their cognate antigen. Lymphocytes from all groups responded with the same degree of proliferation (**Fig. 6 C**). The full susceptibility of IL-17F$^{-/-}$ mice to EAE could have resulted from a compensatory increase in the production of IL-17A. However, IL-17F$^{-/-}$ lymphocytes showed a consistent decrease in the production (**Fig. 6 A**) and frequency (**Fig. 6 B**) of IL-17A secreting cells. Even upon overwhelming *in vitro* polarization towards the T_H17 lineage the proportion of IL-17A producing cells as much as the amount of cytokine produced decreases drastically in IL17F-targeted cells, when compared to wild type T cells (**Fig. 4 D-E**). While our data indicate some degree of haplo-insufficiency of the IL-17F allele (**Fig. 6 E and Supplemental fig. 1 E**), targeting this locus has a profound inhibitory impact on IL-17A expression.

Discussion

After the discovery of distinct T cell polarization patterns by Mosmann and colleagues (162), it was accepted for more than a decade that IFN-γ secreting T_H1 cells are the main encephalitogenic population in CNS inflammatory diseases such as MS and EAE. In addition, T_H1 promoting factors such as IL-12 and IL-18 were considered indispensable for the initiation of autoimmune disease in mice. This simplistic paradigm had to be revised when it was discovered that mice deficient in IFN-γ, TNF-γ, IL-12p35 and IL-18 are either fully EAE-susceptible or hyper-susceptible. While IL-12 is dispensable for the induction of EAE, its close relative IL-23 has been demonstrated to be absolutely essential. Langrish *et al.* initially were able to show that IL-23 induces the secretion of IL-17A by effector T cells (156). IL-17A secreting effector T cells were ultimately termed T_H17 cells and are now established as a distinct helper T cell subset. In addition, the close association of T_H17 cells with inflammatory autoimmune diseases such as RA, MS and psoriasis has clearly marked this population as pathogenic (155). Several reports have then elucidated the conditions

to polarize towards this lineage *in vitro* (109,155).

While IL-17A is now considered to be the main driving force behind tissue inflammation, to this day, virtually all claims are based on a correlative relationship between T_H17 cells and their presence in an inflammatory lesion. Assuming that IL-17A drives the inflammatory process and could contribute to blood brain barrier breakdown and increased detrimental neutrophil activity, we generated transgenic mice in which T cells produce high levels of IL-17A. Surprisingly however, drastically increased levels of IL-17A expression neither visibly impact on the development of EAE nor on the quality and quantity of inflammation in the CNS. Komiyama *et al.* previously addressed the role of IL-17A in EAE by generating a deficient mouse strain (130). Unlike the deficiencies in IL-23 and IL-6 which render mice completely resistant to EAE, loss of IL-17A only marginally impacts on the severity of disease. The far more critical role of IL-23 and IL-6 in the development of autoimmune inflammation indicates that the associated disruption of IL-17A and -17F production is a symptom but not the cause of the EAE resistance observed in IL-23 and IL-6-deficient mice. Matching our conclusions, also in other models of autoimmunity such as experimental autoimmune uveoretinitis (EAU), the function of IL-17A appears to be redundant (163). Luger *et al.* recently demonstrated that while IL-17 can participate in the pathogenesis of EAU, it has by no means an essential role (164). The concept that IL-17A itself is not likely the only pathogenic molecule generated by T_H17 cells was further supported by the report of McGeachy *et al.*, who could demonstrate that IL-23 driven T_H cells, but not TGF-β/IL-6 driven T_H cells were encephalitogenic, regardless of their secretion of IL-17A (115).

By now, a whole family of new cytokines has been grouped around IL-17A, all sharing a distinct structural feature, a 4-cystein knot (165). The closest associate to IL-17A is IL-17F (158,159). Both cytokines are functionally related to neutrophil recruitment and expansion, angiogenesis, tissue remodeling and the induction of pro-inflammatory factors, like IL-1β, TNF-α, chemokines and defensins (139,157,166).

We found that the loss of IL-17A coincides with a significantly elevated level of IL-17F expression, making IL-17F an ideal candidate to compensate for the loss of IL-17A in gene-targeted mice. We generated IL-17F deficient mice and discovered that they are also fully susceptible to EAE and have no appreciable defect in generating an inflammatory response. One could argue conversely, that in IL-17F$^{-/-}$ mice IL-17A could compensate and that the true function of IL-17A and IL-17F can only be appreciated in mice lacking both genes. Unfortunately, the close proximity of the loci of these two cytokines makes it virtually impossible to obtain double-deficient mice by mere interbreeding (ca. 44 Kb in between the two genes). However, while IL-17A$^{-/-}$ T cells have the propensity to secrete much more IL-17F upon activation, our IL-17F-targeted T cells make significantly less IL-17A than wild type mice, which was also observed in a different IL-17F deficient mouse strain, generated

by insertion of an transcriptional stop into exon 2 (167). Our current hypothesis is that either the gene-targeting of IL-17F interferes with the promoter/enhancer activity of IL-17A or that IL-17F exerts some kind of regulatory effect on IL-17A production In any case, the fact that IL-17F$^{-/-}$ mice show this drastic reduction in IL-17A strongly supports the notion that neither IL-17A nor IL-17F, either individually or in combination, are essential for the development of autoimmune CNS inflammation. In addition, the fact that mice in which transgenic overexpression of IL-17A is directed towards T cells also display an unaltered EAE phenotype, further eliminates IL-17A as an important player in CNS-autoimmunity. Considering that IL-17A, as a pro-inflammatory cytokine, is produced in large amounts at sites of inflammation in the brain and elsewhere it is expected to find some degree of symptomatic attenuation. Integrating all current data generated by deletion of IL-17A as much as IL-17F and IL-22 or induced overexpression of IL-17A by critical evaluation of its statistical vs. biological relevance we must conclude that, unlike IL-23, none of those T_H17 cytokines are key players in autoimmune disease like EAE.

The mere presence of a pro-inflammatory molecule at the inflammatory site is often interpreted to convey a vital function ultimately to be translated into an attractive drug target. However, while we can clearly confirm that IL-17F has all the features of such a factor, *in vivo* targeting shows that this correlation is not in fact causative. While IL-17 family member molecules currently serve their purpose as markers for pathogenic self-reactive cells, we conclude that other, thus far unidentified factors or mechanisms employed by T_H17 cells must convey their pathogenic capacity. Alternatively, it is also possible that T_H17 cells harbor a small population of IL-23-driven T cells which possess all the pathogenic potential, and that T_H17 cells have gained their place in the spotlight based upon the concept “guilty by association”.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Harlan Laboratories. IL-17A$^{-/-}$ mice were generously provided by Dr. Yoichiro Iwakura (University of Tokyo, Japan). IL-17F$^{-/-}$ (129xC57BL/6) and IL-17A$^{ind/+}$ mice were generated as described in Supplemental Figure 1. Animal experiments were approved by the Swiss Veterinary Office (13/2006, Zurich, Switzerland) and the Central Animal Facility Institution of the University of Mainz. EAE induction and monitoring of disease severity was performed as previously described (154).

In vitro assays

Lymphocytes were cultured in RPMI1640 containing 10% FCS (both from Invitrogen). Restimulation of primed lymphocytes from lymph node, spleen or cerebellum was measured on 7 (pre-EAE) dpi by MOG_{35-55}/CFA (154) and challenged with 50µg/ml MOG_{35-55} or 5µg/ml ConA. Proliferation was assessed by thymidin incorporation (168) and cytokine release by ELISA (BD Pharmingen) and ELISPOT (154) as described. Activating anti-CD3 and anti-CD28 antibodies used in concentration of 1µg/ml and 6ng/ml, respectively. Fluorocytometric analysis of surface marker expression was performed as described (36). Intracellular cytokine staining was performed with the Cytofix/Cytoperm™ Plus Kit (BD Bioscience) according to manufacturer's directions. Antibodies used: anti-IL-17A (TC11-18H10), anti-IFN-γ (XMG1.2) (BD Pharmingen) and anti-IL-17F (R&D Systems). The IL-17F antibody was labeled using the Alexa Fluor® 488 Monoclonal Antibody Labeling Kit (Invitrogen).

T cell polarization

For the *in vitro* generation of T_H17 cells, splenocytes were harvested 7 dpi with MOG/CFA and were restimulated with 20 µg/ml MOG_{35-55} , 5ng/ml TGF-β, 20 ng/ml IL-6 (both from PeproTech EC, UK), 10 ng/ml IL-23 (R&D Systems), 5 µg/ml anti-IFNγ (R4-6A2) (Bioexpress) and 5 ng/ml IL-2 (eBioscience). Anti-IFN-γ was added daily, IL-23 on day 0 and 2. Cells were analysed on day 6 post culture.

Real-time RT-PCR

RNA was extracted and cDNA prepared as described. The primers used for IL-17F were: fw: CTGTTGATGTTGGGACTTGCC and rev: TCACAGTGTTATCCTCCAGG . β-actin and IL-17A primers were described elsewhere (154).

Histology and immunohistochemical staining

Spinal columns were fixed in 4% paraformaldehyde in PBS, paraffin-embedded, cut and stained with hematoxylin-eosin (H&E) according to standard protocols. Immunohistochemical stainings on serial sections using antibodies to CNPase (1:500, Chemicon) and glial fibrillary acidic protein (GFAP, 1:4000, DAKO) were carried out on an automated Benchmark staining apparatus (Ventana Medical Systems), following the manufacturer's guidelines.

Extraction of mononucleated cells from inflamed CNS tissue and subsequent cytofluorometric analysis was performed as described previously (36).

Statistical analysis

Clinical development was evaluated using a two-tailed Student's T test as well as ANCOVA analysis using the R project for statistical computing.

Acknowledgments

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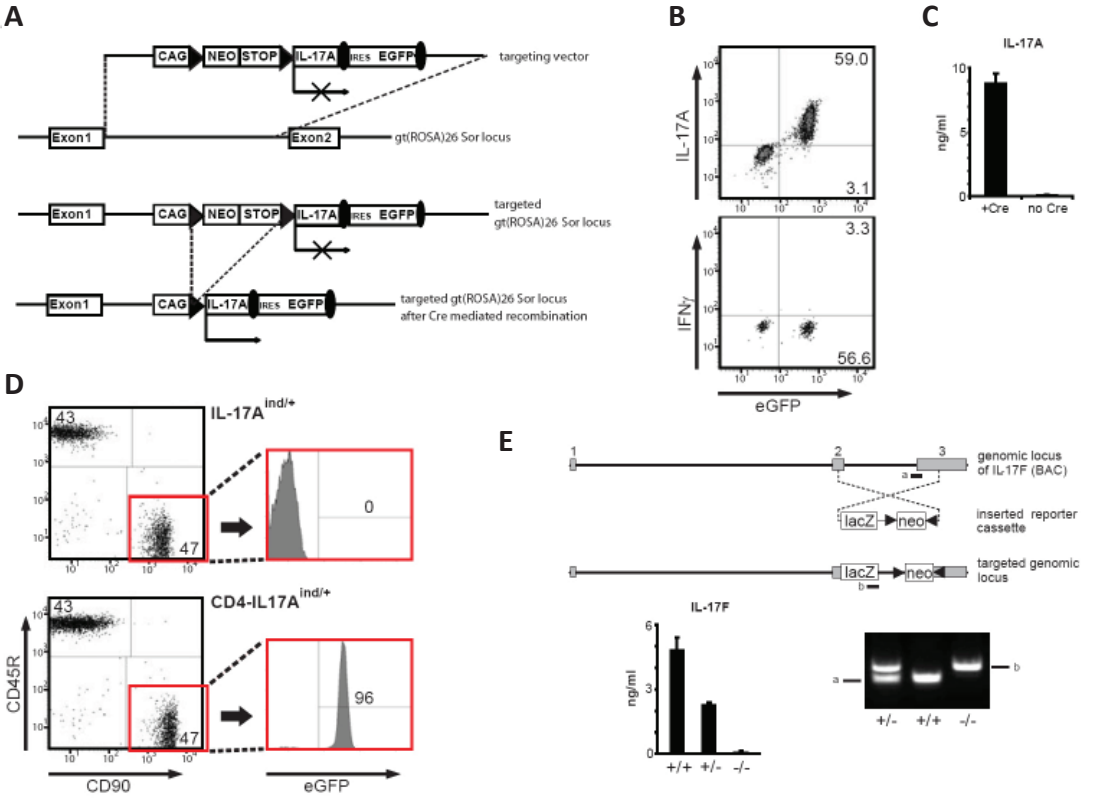


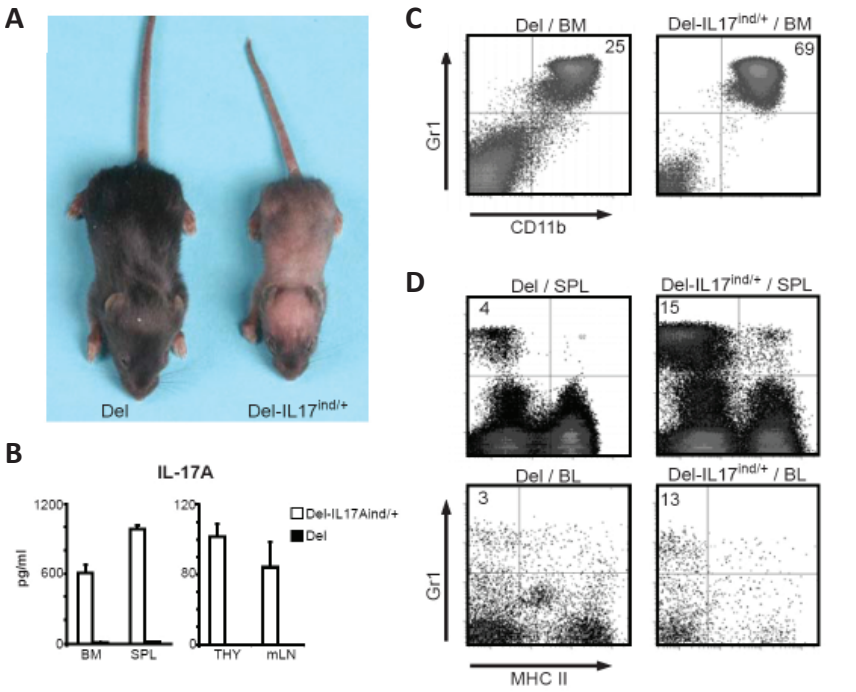
Supplemental Figure 1: Generation of the IL-17A overexpressing and IL-17F deficient mouse strains.

(A) Generation of the IL-17A$^{ind/+}$ allele was carried out using homologous recombination in embryonic stem cells (C57BL/6-Bruce 4). Our conditional ‚knock-in' approach introduced the targeting construct into the endogenous gt(ROSA)26Sor locus. Upon Cre-mediated recombination, a lox-P-flanked transcriptional STOP cassette is excised 5' of an IL-17A CDS insert and an IRES-EGFP element, allowing a dual expression of IL-17A and EGFP under the control of the chicken β-actin (CAG) promoter. (B) 1x10^6 whole lymph node cells from IL-17A$^{ind/+}$ mice were cultured for 4 h with or without (data not shown) Tat-Cre protein to induce IL-17A and EGFP expression in the presence of anti-CD3, anti-CD28 and IL-2. After 36 h, cells were briefly cultured with Brefeldin A and stained for CD4 and either IL-17A or IFN-γ. Percentages of IL-17A$^+$EGFP$^+$ and IFN-γ$^+$EGFP$^+$ cells are given in the quadrants. No EGFP was detected in wild type mice also treated with Tat-Cre (data not shown). (C) LN cells were cultured as in B, and supernatants extracted after 36 h. IL-17A secretion was measured by ELISA. (D) EGFP expression was measured in T cells populations after crossing the IL-17Aind allele to the CD4 Cre transgene. EGFP expression was clearly detectable in CD90$^+$ splenocytes from CD4-IL17A$^{ind/+}$. No EGFP was detectable in IL-17A$^{ind/+}$ littermates. (E) IL-17F gene-targeting strategy for the generation of the IL-17F-deficient mouse strain. A reporter gene/resistance cassette (lacZ gene and a Ub1-Em7 promoter driven neomycin resistance gene, neo, flanked by loxP sites) was introduced into il17f exon 2 and 3 of a BAC containing the IL-17F gene locus. 129S6SvEv/C57B/6F1 embryonic stem cells were targeted with a BAC carrying the replaced il17f gene locus and were subsequently screened as described elsewhere (28). "a" and "b" represent amplicons for the wt and targeted allele respectively. Complete loss of IL-17F in the IL-17^{null} mouse was confirmed by ELISA on supernatant of T_H17 polarized splenocytes.

Supplemental Table I: Homeostatic leukocyte composition in spleen and thymus of naïve IL-17F deficient and control mice.

Spleen	Percentage		
	+/+	+/-	-/-
T_H cells (CD4$^+$)	14	16	14
CTL (CD8$^+$)	20	21	23
T_{Reg} cells (FoxP3$^+$)	3	3	2
NK cells (CD3$^-$, NK1.1$^+$)	3	4	3
NK T cells (CD3$^+$, NK1.1$^+$)	2	3	2
B cells	38	38	37
Macrophages (CD11b$^+$)	4	4	4
DC (CD11c$^+$)	4	4	3

Thymus	Percentage		
	+/+	+/-	-/-
T_H (CD4$^+$)	5	6	4
CTL (CD8$^+$)	14	15	13
Double positive T_H cells	78	78	80



E

BLOOD	Del-IL17Aind/+			Del		
WBC (white blood cells) ($\times 10^3$ cells/µl)	4.20	1.77	1.35	1.53	1.50	3.63
RBC (red blood cells) ($\times 10^6$ cells/µl)	6.18	5.34	3.45	4.36	8.74	7.98
HGB (hemoglobin) (g/dl)	9.00	7.80	8.00	14.20	14.60	12.60
HCT (hematocrit) (%)	31.20	27.30	29.50	46.00	47.20	44.80
MCV (mean corpuscular volume) (fL)	50.40	50.90	46.10	53.90	54.00	56.00
MCH (mean corpuscular hemoglobin) (pg)	14.50	14.60	12.50	16.20	16.70	15.70
MCHC (mean corpuscular hemoglobin conc.) (g/dl)	28.70	28.70	27.00	30.00	30.90	28.00

HGB	P= 0.0015	***
HCT	P= 0.0002	***
MCH	P= 0..0347	*
MCV	P= 0.0301	*

Supplemental Figure 2: Del-IL-17Aind mice display increased granulocyte production and accumulation.

(A) (Del = Deleter Cre, and Del-IL-17A^{ind} =Deleter Cre x IL-17A^{ind} mice) Mice of two weeks of age from Del-IL-17A^{ind} versus Del control present with stunted growth, thin coats, psoriatic skin and blindness. (B) 1x10^6 cells from bone marrow (BM) and spleen (SPL), and 2.5x10^5 cells from mesenteric lymph nodes (MLN) and thymus (THY) were cultured for 24h in cell culture medium the absence of any stimulation. Resulting supernatants were assayed for IL-17A secretion by ELISA. Error bars represent SEM. (C) Bone marrow (BM) from 4 week old Del-IL-17A^{ind} mice was stained using anti-GR1 and anti-CD11b and analysed by FACS. Percentages are given in the representative quadrant, and represent 6 mice in three independent experiments. (D) Spleen (SPL) and blood (BL) samples from Del-IL-17A^{ind} and littermate control mice were stained with antibodies directed against GR-1 and MHC-class II. Percentages are given in the representative quadrants, and represent either 8 mice in three independent experiments (SPL) or 5 mice in two separate experiments (BL). (E) Blood analysis of three Del-IL-17A^{ind} and Del control mice. Statistical analysis of the averages by Student's t-test.

IL-22 is expressed by T_H17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis

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Abstract

Lately, IL-17 secreting T_H cells received an overwhelming amount of attention and are now widely held to be the major pathogenic population in autoimmune diseases. In particular IL-22 secreting T_H17 cells were shown to specifically mark the highly pathogenic population of self-reactive T cells in experimental autoimmune encephalomyelitis (EAE). As IL-17A itself was found to only play a minor role during the development of EAE, IL-22 is now postulated to contribute to the pathogenic function of T_H17 cells. The goal of this study was to determine the role and function of IL-22 during the development of CNS autoimmunity *in vivo*. We found that CNS-invading encephalitogenic T_H17 cells coexpress IL-22 and that IL-22 is specifically induced by IL-23 in autoimmune-pathogenic $CD4^+$ T cells in a time- and dose-dependent manner. We next generated IL-$22^{-/-}$ mice, which - in contrast to the prediction that expression of inflammatory cytokines by CNS-invading T cells inevitably confers pathogenic function – turned out to be fully susceptible to EAE. Taken together, we show that self-reactive T_H cells coexpress IL-17 and IL-22, but that the latter also does not appear to be directly involved in autoimmune pathogenesis of the CNS.

Introduction

Multiple sclerosis (MS) is the most common inflammatory disease of the CNS and its animal model EAE is mediated by the actions of auto-reactive encephalitogenic T_H cells. While T_H1 cells were long suspected to be the major pathogenic population, the discovery that IL-23 and not the T_H1 inducing cytokines IL-12 and IL-18 is vital for EAE development initiated a major paradigm shift with regards to the role of T_H1 cells in inflammation (78,79,99,153,154). The impact of IL-23 on T_H cells appears to be restricted to memory cells, which in response to IL-23R engagement secrete IL-17 (153). IL-17 expression by T cells correlates superbly with an autoimmune pathogenic phenotype and this polarization pattern was coined T_H17 (101,169). While studying IL-23 under various inflammatory conditions lead to the discovery of T_H17 cells, it was later found that the cytokines TGF-β and IL-6 are dominant in their capacity to polarize T_H17 cells (107,109). The role and function of IL-23 in maintaining this phenotype remains a subject of debate (153).

T_H17 cells received much attention lately and mice lacking IL-17A were found to be moderately resistant to EAE (130). However, in contrast to IL-17A$^{-/-}$ mice, IL-23 deficient mice are completely EAE-resistant (78,79). Thus, we reasoned that IL-17A is unlikely to be the only factor produced by T_H17 cells involved in the inflammatory process. To identify the expression signature of IL-23 driven genes, we used high density transcriptomics and identified IL-22 to be induced by IL-23 in autoimmune-pathogenic CD4$^+$ T cells in a time- and dose-dependent manner. IL-22 belongs to the IL-10 super-family of cytokines and exhibits – unlike IL-10 – potent pro-inflammatory properties. Its recently reported role in psoriasis (140,145,146) combined with our finding that IL-22 is specifically induced by IL-23 points towards a relevant function of IL-22 in autoimmune inflammatory diseases. Bettelli and colleagues further reported that IL-22 marks a particularly pathogenic population of autoreactive T cells implicating IL-22 as a major pathogenic cytokine during CNS inflammation (170). In addition the IL-22 gene, together with IL-26 and IFN-γ on the human chromosome 12q14, are considered a prominent susceptibility locus for MS (171). We found that following IL-23 stimulation, IL-22 is specifically secreted by pathogenic T_H cells. To determine the actual role of this cytokine in autoimmune inflammation, we generated IL-22$^{-/-}$ mice, which were found to be surprisingly fully susceptible to EAE. We show that self-reactive T_H cells coexpress IL-17 and IL-22, but that the latter does not appear to be directly involved in autoimmune pathogenesis of the CNS.

Results

IL-23 induces IL-22 gene expression

To elucidate the identity of IL-23-driven gene transcripts, we devised two reciprocal approaches for whole genome transcriptomics. We compared gene-expression induced by IL-23 stimulation with those absent in Ag-driven IL-23 deficient ($p40^{-/-}$) lymphocytes. In the first approach, genes upregulated (more than fourfold) by IL-23 were identified by stimulating splenocytes obtained from an unmanipulated mouse with recombinant IL-23 or IL-12 as a control. In the second approach, we immunized wt, IL-$12p35^{-/-}$ and IL-$12/23p40^{-/-}$ mice with keyhole limpet hemocyanin (KLH) and 7 dpi harvested lymphocytes and re-challenged them *in vitro* with KLH prior to harvesting the mRNA for microchip analysis (Affimetrix chip #MOE430A). We used IL-12 as a control firstly, because IL-12-

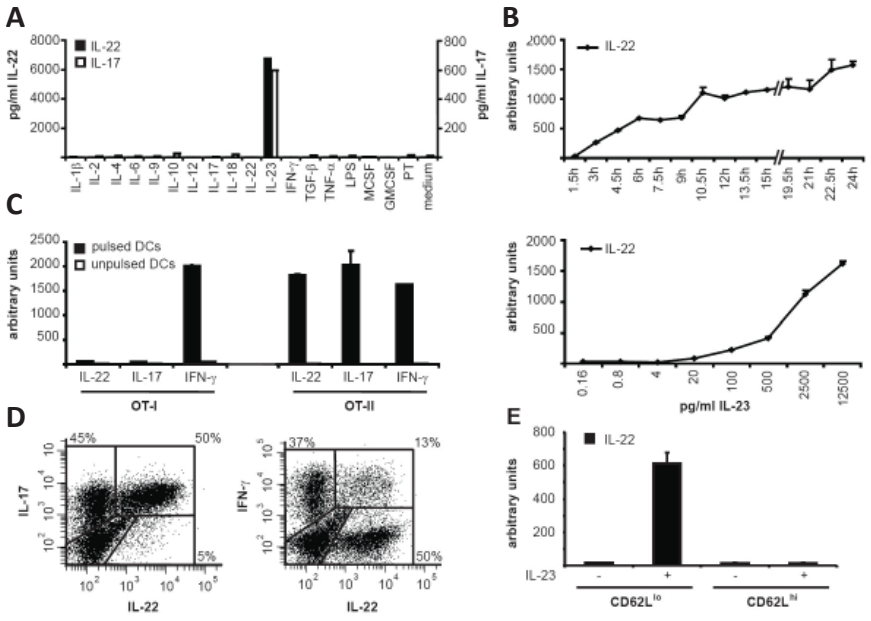

Figure 7: IL-22 expression is specifically induced by IL-23. *(A) $4x10^6$ splenocytes were obtained from C57BL/6 mice, stimulated with 20 ng/ml of the indicated substances and incubated for 24 h. The supernatant was collected and used for IL-22 and IL-17A protein detection by ELISA. (B) $4x10^6$ naïve wt splenocytes were stimulated with 20 ng/ml IL-23 and collected at indicated time points or stimulated with indicated concentrations of IL-23 and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22 mRNA expression performed. (C) $4x10^6$ $CD8^+$ or $CD4^+$ T cells were purified from OT-I or OT-II splenocytes, respectively and incubated with cognate peptide pulsed or unpulsed bone-marrow derived DCs for 48 h and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22-, IL-17- and IFN-γ mRNA expression performed. All data shown are representative of at least three individual experiments and SD were calculated from duplicate wells. (D) Splenocytes from a MOG-immunized C57BL/6 mouse were activated in vitro 7 dpi with 15 µg/ml MOG peptide, 10 ng/ml IL-23, 20 ng/ml IL-6, 5 ng/ml hTGF-β, IL-7 and IL-2 and 5 µg/ml anti IFN-γ ab to generate T_H17 cells. Intracellular cytokine staining was performed on day 5. The plot is gated on $CD45^+CD4^+$ cells. Shown are the percentages only of polarized T cells. (E) Splenocytes were obtained from C57BL/6 mice and MACS-sorted for $CD4^+CD62L^{hi}$ and $CD4^+CD62L^{lo}$. $4x10^6$ cells were stimulated with 20 ng/ml IL-23 and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22 mRNA expression performed. Data shown are representative of three experiments.*

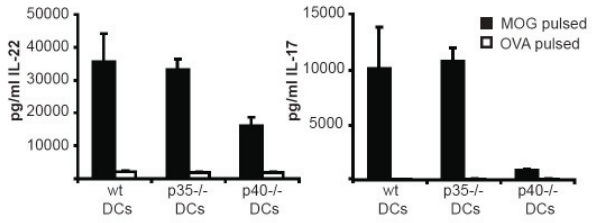

Figure 8: IL-22 and IL-17 are produced by MOG-reactive T cells upon encounter with their cognate Ag.
T cells from mice transgenic for the 2D2 T cell receptor were isolated and stimulated with MOG_{35-55} or control peptide (5 µg/ml) pulsed mature bone-marrow derived DCs obtained from wt-, $p35^{-/-}$- or $p40^{-/-}$-mice for 2d and IL-22 and IL-17A protein expression was measured by ELISA. Shown is a representative of three individual experiments (n=4).

induced gene expression is well characterized and secondly to eliminate IL-12-induced target genes from our analysis. By combining both data sets, we found IL-22 to be specifically and strongly induced by IL-23 (data not shown).

To verify that IL-22 expression is specifically induced by IL-23, we treated splenocytes derived from unmanipulated C57BL/6 mice with an array of different stimuli, harvested the mRNA and measured IL-22 and IL-17A protein expression by ELISA (**Fig. 7 A**). Other than IL-23, none of the used substances elicited significant levels of IL-22 and IL-17 expression in splenocytes after 24 h of stimulation. Different concentrations of the different stimuli were used (data not shown). Our data show that a population of splenocytes present in naïve pathogen-free C57BL/6 mice respond to IL-23R engagement with IL-22 and IL-17 expression. To further characterize the kinetics and dose-dependence of IL-23-induced IL-22 production, we stimulated wt lymphocytes obtained from an untreated mouse with IL-23 for different periods of time or in the presence of different concentrations of IL-23 and observed that IL-22 expression is induced in a time- and dose-dependent manner (**Fig. 7 B**). We observed a similar expression pattern with IL-17 (data not shown). To verify the notion that T_H cells and not CTLs are the main source of IL-22, we stimulated purified $CD4^+$ as well as $CD8^+$ T cells obtained from Ovalbumin TcR Tg mice (OT-II and OT-I respectively) with cognate peptide pulsed DCs for 24 h and found that only TcR Tg $CD4^+$ T cells made IL-22 (**Fig. 7 C**). By intracellular cytokine staining of T_H17 cells, we could show that more than 90% of IL-22-secreting cells also produce IL17, while fewer IFN-γ secreting cells coexpress IL-22 (20%) (**Fig. 7 D**). To identify whether IL-23 stimulates the secretion of IL-22 by naïve or memory T cells, we purified memory T cells ($CD62L^{lo}$) and naïve T cells ($CD62L^{hi}$) followed by an O/N stimulation with IL-23. Our data confirm that IL-23 primarily drives the memory T cell pool and does not influence the naïve pool in regards to cytokine secretion measured (**Fig. 7 E**).

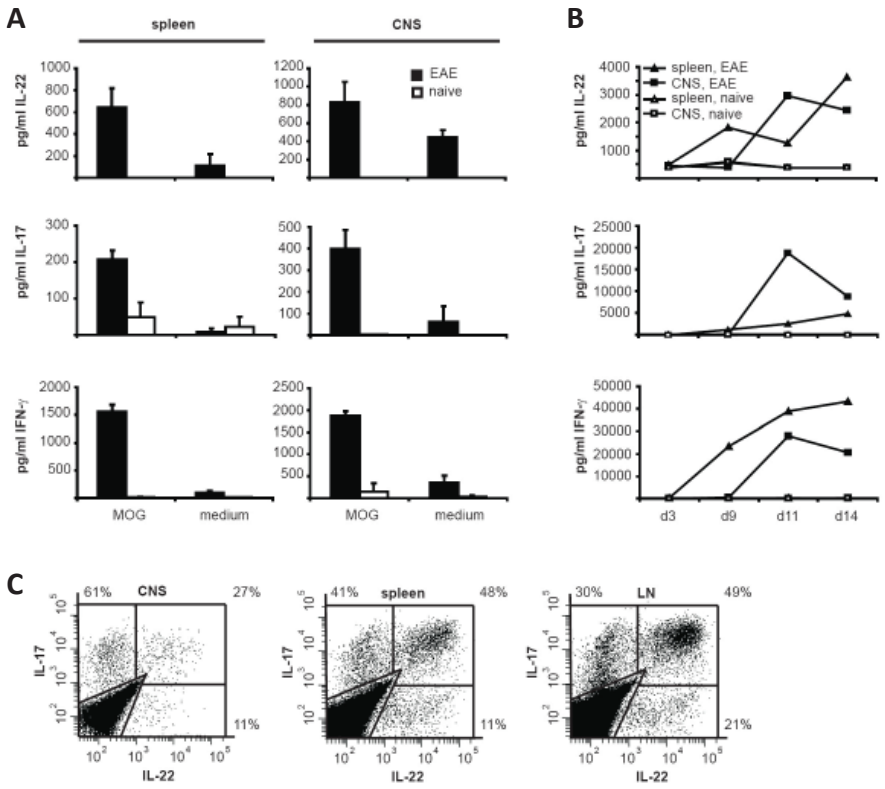

Figure 9: CNS infiltrating T_H17 cells express IL-22.
Wt mice were immunized with MOG_{35-55} emulsified in CFA and sacrificed either (A/C) at the peak of disease (d 21) or (B) at indicated time points after immunization. Lymphocytes were isolated out of CNS and spleen or LN of naïve or MOG_{35-55} immunized wt mice, restimulated with 50 µg/ml of their cognate antigen MOG_{35-55} and IL-22-, IL-17A- and IFN-γ expression was analyzed by A) ELISA or (C) intracellular cytokine staining. (A) Shown is a representative ELISA of four individual experiments (n=2/group/experiment). (B) Pre-onset disease was performed with n=3. (C) Shown are the percentages only of polarized T cells. Shown is a representative of 3 individual experiments n=3.

IL-22 is expressed by encephalitogenic T_H cells

To determine the induction of IL-22 and IL-17A expression in a more physiologic manner in response to cognate Ag, we isolated T cells from MOG-reactive TcR transgenic (2D2) mice and stimulated them with MOG_{35-55} or control peptide-pulsed mature bone-marrow derived DCs obtained from wt-, IL-12p35$^{-/-}$- or IL-12/23p40$^{-/-}$- mice. IL-22 and IL-17A expression was subsequently measured by ELISA. MOG-reactive T cells clearly expressed high levels of IL-22 and IL-17A after encounter with their cognate antigen (**Fig. 8**). This response is dependent on IL-23 as reduced levels of IL-22 and IL-17 were detectable when T cells were co-cultured with DCs obtained from p40IL-12/23$^{-/-}$ mice. We have also performed this restimulation experiment using polyclonal effector T cells isolated from MOG-immunized wt mice and confirmed that the expression of IL-22 and IL-17 is dependent on the secretion of IL-23 by APCs (data not shown). In order to analyze the expression of IL-

22 in mice with autoimmune inflammation, we induced EAE in wt C57BL/6 mice through immunization with MOG_{35-55} emulsified in CFA and harvested brain and spinal cord as well as splenocytes at the peak of disease (d 21). Lymphocytes were isolated as described, challenged with MOG_{35-55} and analyzed for cytokine-expression after 48 h by ELISA (**Fig. 9 A**). As expected, we found IFN-γ and IL-17 to be expressed by splenocytes and CNS-invading lymphocytes. Most importantly, we detected a significant production of IL-22 by encephalitogenic, CNS-infiltrating lymphocytes after re-encounter with their cognate MOG-antigen *in vitro.* Kinetic analysis of IL-22 secretion was performed by sacrificing mice at different time points after immunization with MOG/CFA. Similar to IFN-γ and IL-17, IL-22 expression by CNS-infiltrating lymphocytes increased with disease severity (**Fig. 9 B**). To study which population of polarized T_H cells secrete IL-22 in peripheral organs and the inflamed CNS, we immunized C57BL/6 mice with MOG/CFA and harvested spleen, LNs and CNS at the peak of clinical EAE (average score of 3). The mononuclear cells were then restimulated with MOG_{35-55} followed by intracellular cytokine staining. Cytofluorometric analysis revealed that in spleen and LN, there is a high overlap of IL-17 and IL-22 secreting T cells, while in the inflamed CNS, IL-17 secreting cells dominate over IL-22 and IL-17/22 secreting T cells (**Fig. 9 C**).

Spleen	percentage	
	wt	IL-22$^{-/-}$
T_H cells (CD4$^+$)	18	16
CTLs (CD8$^+$)	11	10
double positive T cells (CD4$^+$CD8$^+$)	0.5	0.5
Tregs (FoxP3$^+$)	0.5	0.4
NK cells (CD3$^-$NK1.1$^+$)	2	1.9
NKT cells (CD3$^+$NK1.1$^+$)	2.1	1.9
B cells (B220$^+$)	62	65
Macrophages (CD11b$^+$)	4	3
DCs (CD11c$^+$)	3	3
naive T cells (CD3$^+$CD62L$^+$)	25	23
memory T cells (CD3$^+$CD62L$^-$)	5	6

LN	percentage	
	wt	IL-22$^{-/-}$
T_H cells (CD4$^+$)	37	37
CTLs (CD8$^+$)	27	27
double positive T cells (CD4$^+$CD8$^+$)	1.2	1.7
Tregs (FoxP3$^+$)	0.3	0.2
NK cells (CD3$^-$NK1.1$^+$)	0.5	0.8
NKT cells (CD3$^+$NK1.1$^+$)	0.4	0.6
B cells (B220$^+$)	33	35
Macrophages (CD11b$^+$)	3	3
DCs (CD11c$^+$)	1	1
naive T cells (CD3$^+$CD62L$^+$)	64	62
Memory T cells (CD3$^+$CD62L$^-$)	9	9

Tables II-IV: Distribution of cell types of the immune system in C57BL/6 and IL-22$^{-/-}$ mice.
Spleens, LNs and thymi were obtained from unmanipulated C57BL/6 and IL-22$^{-/-}$ mice, cells were isolated and stained with flouro-chrome conjugated abs against the indicated cell-type specific surface markers. Data shown are representative of three individual experiments.

thymus	percentage	
	wt	IL-22$^{-/-}$
T_H cells (CD4$^+$)	7	7
CTLs (CD8$^+$)	3	3
double positive T cells (CD4$^+$CD8$^+$)	87	83

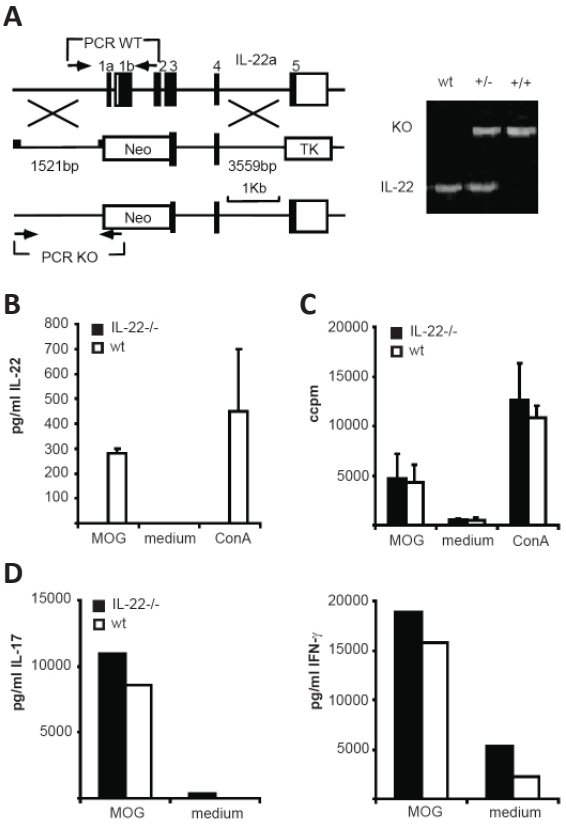

Figure 10: Generation and characterization of IL-22$^{-/-}$ mice.
(A) The structure of the IL-22α locus, the targeting vector, and the predicted homologous recombination are shown. The exons are shown as boxes, with white and black boxes for non coding and coding regions respectively. The size of the 5'and 3' arms, as well as the location of the primers used for genotyping are indicated. Neo, neomycin resistance cassette, TK, thymidine kinase cassette. For genotyping, the wt and targeted alleles were amplified from F_2 tail genomic DNA as described in material and methods. Lymphocytes were isolated out of spleen of MOG_{35-55} immunized wt or IL-22$^{-/-}$ mice, restimulated with 50 µg/ml of their cognate antigen MOG_{35-55} or 5 µg/ml ConA. (B) IL-22 expression was analyzed by ELISA, (C) proliferation was measured by thymidin uptake (shown is a representative of 3 individual experiments n=3) or (D) IL-17A and IFN-γ expression was analyzed by ELISA. Data shown are representative of at least three individual experiments and SD were calculated from duplicate wells.

Gene targeting of IL-22 does not prevent EAE development

Given the clear expression pattern of IL-22 associated with pathogenic T_H17 cells, we sought to investigate whether IL-22 plays a role in inflammation of the CNS. To do so, we generated IL-22$^{-/-}$ mice by replacing the coding-exons 1a, 1b, 2 and a part of exon 3 of the IL-22 gene with a neomycine resistant gene (**Fig. 10 A**) and verified the absence of IL-22 by genomic PCR, RT-PCR and ELISA (**Fig. 10 A, B** and data not shown). The mice do not display any obvious malformation of the hematopoietic system and developed normally (**Tables II-IV**). When we analyzed the proliferating capacity as well as the cytokine expression profile of IL-22$^{-/-}$ cells after re-encounter with MOG_{35-55} by thymidin incorporation or ELISA, respectively, we observed that they behaved similar to wt cells (**Fig. 10 C, D**).

We induced EAE in the IL-22$^{-/-}$ mice through immunization with MOG/CFA and recorded the clinical disease development. In spite of our expectations that IL-22 would display

genotype	wt	IL-22$^{-/-}$
incidence	21 of 22	19 of 19
Day of disease onset	13.3 ± 1.78	13.7 ± 2.22
Max. clinical score	2.93 ± 0.44	3.17 ± 0.46

Table V: IL-22$^{-/-}$ are fully susceptible to EAE.
IL-22$^{+/+}$ and IL-22$^{-/-}$ mice were immunized with MOG_{35-55} emulsified in CFA and the clinical score recorded as described. Three merged representative experiment (n≥19) of five individual experiments are shown. Onset and max. clinical score is calculated ± Average deviation. Statistical significance was determined using an unpaired Student's T-test.

pro-inflammatory encephalitogenic properties, we observed that IL-22$^{-/-}$ mice developed disease similar to wt controls (**Fig. 11 A & Table V**). Cytofluorometric analysis of CNS invading mononucleated cells revealed that their numbers and subset-distributions are indistinguishable between the inflamed CNS of wt and IL-22$^{-/-}$ mice (**Fig. 11 B**). Histological analysis of spinal cord cross-sections further displayed similar inflammatory lesions consisting of activated Iba1^{+} macrophages/microglia, CD3^{+} T cells and few B220^{+} B-cells resulting in an impairment of myelinated and axonal structures (**Fig. 11 C** and data not shown). Therefore, while IL-22 is clearly expressed by encephalitogenic T_H17 cells, it does not appear to be crucial for the development of autoimmune inflammation of the CNS.

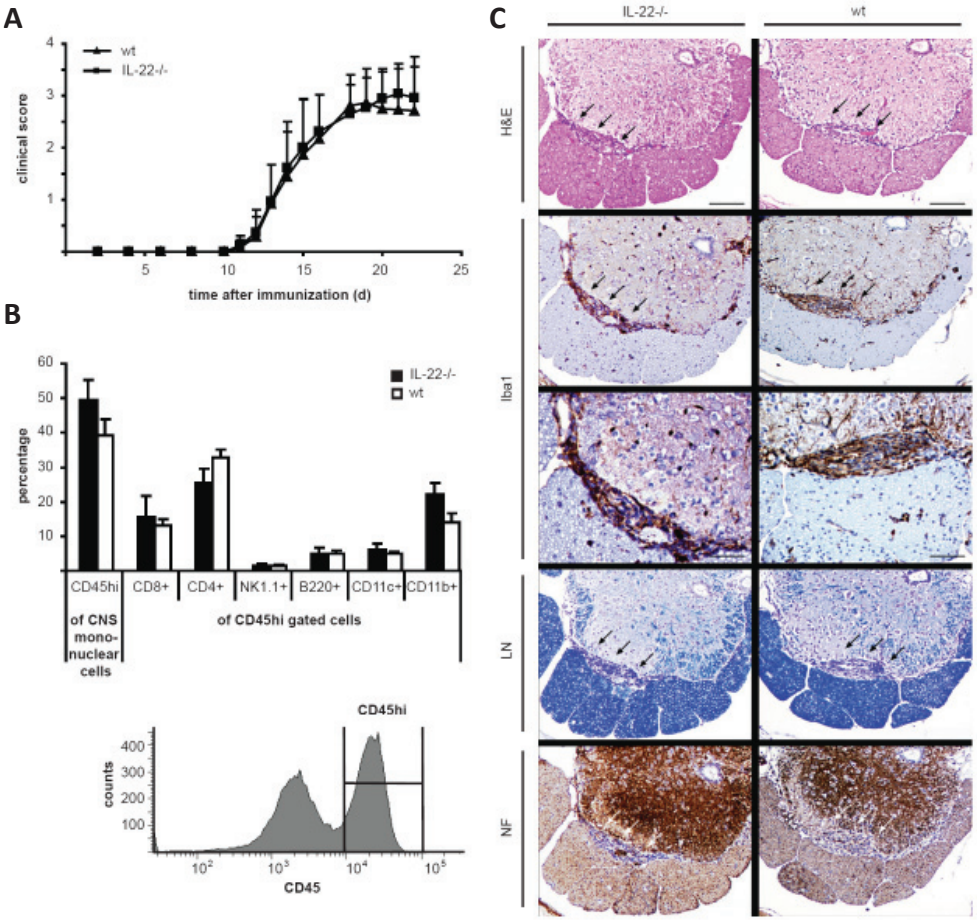

Figure 11: IL-22$^{-/-}$ mice are fully susceptible to EAE.
(A) IL-22$^{+/+}$ and IL-22$^{-/-}$ mice were immunized with MOG$_{35-55}$ emulsified in CFA and the clinical score recorded as described. Two merged representative experiment (n≥12) of five individual experiments are shown. Error bars represent the average deviation. (B) Mice were sacrificed at the peak of disease (d 22), lymphocytes were isolated out of CNS, stained for the indicated surface markers and analyzed by flow cytometry. The histogram represents a staining for CD45 to distinguish CNS resident microglia (CD45^{lo}) from CNS-invading leukocytes (CD45^{hi}). Shown is an average of 5 individually analyzed mice ± Average deviation. (C) Spinal cord cross-sections of wt and IL-22$^{-/-}$ mice displayed similar inflammatory lesions (arrows), which often appeared to be accentuated around vessels. They consisted of many activated Iba1^{+} macrophages/microglia (2nd and, at higher magnification, 3rd row) and caused an impairment of myelinated and axonal structures, as depicted by a Luxol-Nissl (LN, 4th row) as well as a neurofilament (NF) stain (5th row). Scale bars: 100 µm for 1st, 3rd, 4th and 5th column, 100 µm for 2nd, 4th and 6th column.

Discussion

In the recent past, the function, origin and regulation of IL-17 expressing T_H cells received much attention by the immunology community. The discovery of this T_H polarization profile (T_H17) finally does resolve a number of conflicting findings regarding the $T_H1/2$ paradigm of tissue directed autoimmune disease (153). The expression of IL-17 by T_H cells correlates extremely well with their pathogenicity during autoimmunity.

However, in spite of this close correlation (99,101,169), several questions regarding their actual effector function remain unanswered. Foremost, the fact that IL-23 is absolutely vital for the development of autoimmune disease, whereas IL-17A alone has only a moderate impact (130), raises the question whether additional thus far unidentified IL-23-driven cytokines have pathogenic properties. We sought to resolve this question by the global analysis of IL-23-induced genes in lymphocytes. We discovered IL-22 to be the most prominent gene expressed by T_H cells after IL-23 treatment. We further found that self-reactive T_H cells required the presence of IL-23 for IL-22 production and that IL-23-deficient APCs were not able to properly induce IL-22 by stimulation of a population of MOG-reactive T cells. In agreement with Liang *et al.*, we found IL-22 to be highly expressed by T_H17 cells (139). This suggested that IL-22 could potentially serve a pathogenic function during EAE. To this end, we performed a longitudinal analysis of IL-22 expression during EAE and found a strong correlation between T cell pathogenicity and IL-22 secretion. Bettelli *et al.* (170) recently claimed that IL-22 expression 'marks' a highly pathogenic and proinflammatory population of autoaggressive T cells, heavily implicating IL-22 to exert a pathogenic function during EAE. Also, the receptor for IL-22, a heterodimer of the IL-10R2 and IL-22R1, like the IL-17A receptor is found primarily on stroma- cells including endothelial cells, epithelials cells and CNS-resident astrocytes (146,170,172). The close association of IL-22 and IL-17 in pathogenic T_H cells, their inducibility by IL-23 and the fact that their receptors are expressed by similar cell types, implies that IL-22 too serves a pro-inflammatory pathogenic role in CNS inflammatory disease.

In order to determine whether IL-22 actually contributes to the development of EAE or whether the crisp correlation between IL-22 expression and encephalitogenicity is only an epiphenomenon, we generated IL-$22^{-/-}$ mice by gene-targeting. To our surprise, we discovered that IL-$22^{-/-}$ mice develop EAE with the same severity, day of onset and clinical manifestations as wt mice. This finding clearly dismisses IL-22 as a major pathogenic player in the development of autoimmune CNS inflammation. The function of IL-22 in autoimmunity however cannot be dismissed altogether. Wolk *et al.* reported that elevated levels of IL-22 can be found in the blood of psoriatic patients and ear-skin acanthosis and inflammation induced by the application of IL-23 is slightly decreased when IL-22 is

absent (140,145).

Taken together, the notion that a cytokine is considered to have pathogenic functions cannot be based on its mere presence in a potentially pathogenic population of T cells. This line of thought had lead to a biased interpretation of the role and function of T_H1 and T_H2 cells in the context of autoimmune disease (153,173-175). We were able to identify such a pro-inflammatory factor, namely IL-22, which is like IL-17A closely associated with an encephalitogenic phenotype. However, the fact that IL-$22^{-/-}$ mice develop severe EAE indicates that IL-22, just like IFN-γ, is not among the pro-inflammatory factors mediating the tissue damage seen in EAE. The requirement of the transcriptions factors which drive T_H1 and T_H17 polarization (T-bet and ROR-γt respectively) indicates that features, other than the main cytokines produced, are responsible to their pathogenic behavior. While T_H17 cells secrete IL-17 as well as IL-22, the report by Kebir *et al.* (176) suggests that cytolytic enzymes and factors that alter the integrity of the blood-brain-barrier may be responsible for the encephalitogenicity of human $T_H17/22$ cells. It is however likely that amongst the genes expressed by T_H17 cells, a number of them may turn out to serve as biomarkers if not therapeutic targets in the treatment of autoimmune diseases in general and MS in particular.

Materials and Methods

Peptides, Antibodies and recombinant cytokines

$MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) were obtained from Research Genetics (Huntsville, Alabama, USA). All recombinant cytokines were purchased from PeproTech and all antibodies were purchased from BD Biosciences. The antibody to murine IL-22 was kindly provided by Genentech, Inc. and labeled with Alexa 488 (Invitrogen) according to the manufacturer's directions.

Mice and induction of EAE

C57BL/6 mice, IL-12 $p35^{-/-}$ and IL-12 $p40^{-/-}$ mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, Maine) and were bred under specific pathogen-free conditions. The 2D2 (MOG-TCR-Tg) mice were provided by V. Kuchroo (Harvard Medical School, Boston, Massachusetts). IL-$22^{-/-}$ mice were generated by targeting exons 1-3 and backcrossed onto C57BL/6 for more than 8 times. The targeting vector was constructed to replace the exons 1a, 1b, 2 and a part of exon 3 of the IL-22a gene by a neomycine resistant gene. A 5'arm of 1521 base pairs was amplified using a mutated sense

primer with a Xho1 site 5'-CTTCGGCTCGAGATGGCCAC-3' a mutated antisense primer containing also a Xho1 site 5'-GCCCTCGAGACACCAGGGTT-3' to allow the direct insertion into the pPNT vector. The 3'arm consisted of a 3559 bp Kpn1 fragment, containing the end of exon 3 and exon 4 was cloned. For genotyping, the targeted gene was amplified using a sense primer located upstream the 5'arm: 5' CTGCTGTCCAACAGAGCTCT-3' and antisense primer on neomycine gene: 5'-CGCCTCCCCTACCCCGGTAGA-3', resulting in a 1.7 kb amplified sequence. The wild type gene was amplified using a sense primer located into the 5'arm 5'-AATCTATGAAGTTGGTGGGA-3' and an antisense primer located on exon 2 5'-ACTGACTCCTCGGAACAGTT-3', resulting in a 1.2 kb amplified sequence. Mouse IL-22 RT-PCR was performed as previously described (131). EAE was induced and scored as described (36).

Histology and Flow cytometry

Whole mouse brains or spinal columns were fixed in 4% paraformaldehyde in PBS, paraffin-embedded, cut and stained with hematoxylin-eosin (H&E) and Luxol-Nissl (LN) according to standard protocols. Immunohistochemical stainings on serial sections using antibodies to neurofilament protein (NF, 200 kD subunit, 1:20, Bio-Science), Iba1 (1:100, Wako Chemicals), CD3 (1:150, Labvision) and B220 (1:1000, Becton Dickinson) were carried out on an automated Nexus staining apparatus (Ventana Medical Systems), following the manufacturer's guidelines. CNS infiltrating lymphocytes were isolated as described previously (154). For flow cytometry, antibodies were incubated with cells for 20 min at 4 °C and then cells were analyzed with a FACSCalibur (BD Pharmingen) and FACSDiva software. Post-acquisition analysis was done with FACSDiva (BD Pharmingen) or FlowJo7 software (Tree Star, Inc.) For intracellular cytokine staining, cells were restimulated with 50 ng/ml PMA, 500 ng/ml ionomycin and GolgiPlug (BD Biosciences) for 5 h. Cells were first stained for surface antigens and then permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's recommendations. Intracellular cytokine staining was performed using antibodies to IFN-γ, IL-17A or IL-22 as described above.

Cell culture and *in vitro* assays

Mice were sacrificed using CO_2, axillary and inguinal lymph nodes (LN) and spleens were collected and treated with 0.5mg/mlDNase and 1mg/ml Liberase (Roche) for 30 min at 37°. Cells were cultured in RPMI supplemented with 10% FCS, 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco) in the presence or absence of the factors indicated in the figure legends and harvested at indicated time points. Where indicated, T cells were purified from splenocytes by magnetic cell sorting with MACS® Beads following the

manufacturer's recommendation (Miltenyi Biotec). BM-derived DCs were generated as described (154). To mature DCs, 10μg/ml LPS (Fluka) was added to the culture for 24h. Mature DCs were pulsed with 5 μg/ml peptide for 4 h, washed extensively and incubated with splenocytes at a ratio of 1:4 and harvested after 48 h.

Cytokine analyis

Elisa for IL-17A (BD Pharmingen, La Jolla) and IL-22 (Antigenix, Huntington Station, NY) were preformed according to the manufacturer's instructions. Proliferation of MOG-reactive cells were stimulated in triplicate for 48 h with either 50 μg/ml of $MOG_{35–55}$, 5 μg/ml of concanavalin A, or medium and 0.5 μCi/ml of [^{3}H]thymidine was added after 24 h for assessment of proliferative responses. Thymidine incorporation was assessed with a Filtermate Collecter (Applied Biosystems) and a scintillation and luminescence counter.

Real-time RT-PCR

Cells or tissues were homogenized in 1 ml Trizol reagent (Invitrogen). Total RNA was extracted and reverse transcription performed using random hexamer primer and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). After PCR amplification using SYBR Green PCR master mix (Invitrogen), quantitative values of each sample were normalized to its β-actin content and converted to relative cDNA quantities by comparison to a standard curve generated with dilutions of β-actin plasmid. Primers were purchased from Operon Technologies (Alameda CA). The primers used were (5'-3') β-actin fw: AGAGGGAAATCGTGCGTGAC, β-actin rev: CAATAGTGATGACCTGGCCGT, IL-22 fw: TTGAGGTGTCCAACTTCCAGCA, IL-22 rev: AGCCGGACGTCTGTGTTGTTA, IL-17 fw: ATCAGGACGCGCAAACATGA, IL-17 rev: TTGGACACGCTGAGCTTTGA.

Acknoledgements

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Human T_H17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation

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Abstract

T_H17 lymphocytes appear to be essential in the pathogenesis of numerous inflammatory diseases. We demonstrate here the expression of IL-17 and IL-22 receptors on blood-brain barrier endothelial cells (BBB-ECs) in multiple sclerosis lesions and show that IL-17 and IL-22 disrupt BBB tight junctions *in vitro* and *in vivo*. Furthermore, T_H17 lymphocytes transmigrate efficiently across BBB-ECs, highly express granzyme B, kill human neurons and promote central nervous system inflammation through $CD4^+$ lymphocyte recruitment.

Results and Discussion

T-helper type 1 (T_H1) (177,178) and type 17 (T_H17) lymphocytes contribute to autoimmune inflammatory diseases(179) including multiple sclerosis and its mouse model, experimental autoimmune encephalomyelitis (EAE) (99,101). Disruption of the BBB and trafficking of autoreactive T cells from the systemic compartment into the central nervous system (CNS) are important, early events in the development of multiple sclerosis lesions (180). In support of T_H1 lymphocytes have been shown to migrate efficiently across the human BBB (181,182). To evaluate T_H17 lymphocyte migration to the brain relative to T_H1 cells, we employed an *in vitro* model of the human BBB using human brain-derived microvascular endothelial cells. We generated human T_H1 and T_H17 lymphocytes *in vitro* using peripheral blood $CD4^+$ lymphocytes cultured with IL-12 and IL-23, respectively. Human T_H17 lymphocytes migrated more avidly across the BBB than did T_H1 or freshly isolated (*ex vivo*) $CD4^+$ lymphocytes (Fig. 12 A, $P < 0.01$). To ensure that the selective accumulation of T_H17 lymphocytes indeed reflects the preferential transmigration ability of T_H17 cells, we analyzed the intracellular cytokine profile of the cell population before and after migration across BBB-ECs, looking specifically at IL-17 and at IL-22, a recently identified cytokine product of T_H17 cells (139,140,183). We noted a significant enrichment in the number of IL-17– and IL-22–expressing $CD4^+CD45RO^+$ memory lymphocytes upon migration across the BBB (Fig. 12 B, $P < 0.001$ for IL-17^+ and $P < 0.05$ for IL-22^+ cells, $n = 3$), confirming the ability of T_H17 lymphocytes to cross the BBB *in vitro*. To further substantiate these observations, we generated myelin oligodendrocyte glycoprotein (MOG)-specific T_H1 and T_H17 lymphocytes from 2D2 mice *in vitro* and transferred these separately into T and B lymphocyte–deficient $Rag1^{-/-}$ mice. Equal numbers of T_H cells were found in the CNS of $Rag1^{-/-}$ mice 7 d after transfer, regardless of whether donor cells were polarized into T_H1 or T_H17 cells, confirming that T_H1 and T_H17 cells primed and expanded in the periphery access the CNS *in vivo* (Fig. 12 C). To validate these human *in vitro* and mouse *in vivo* observations, brain sections from humans with multiple sclerosis and from unaffected controls were immunostained for CD45RO and IL-17 or IL-22. Numerous $CD45RO^+$ cells immunopositive for IL-17 or IL-22 were detected in highly infiltrated multiple sclerosis lesions, but not in normal-appearing white matter or non-inflamed brain specimens (Fig. 12 D, E). Taken together, these results emphasize the potential importance of T_H17 lymphocyte infiltration into the CNS and these lymphocytes' consequent involvement in lesion formation in multiple sclerosis and EAE.

So far, the encephalitogenic activity of T_H17 cells has been attributed to IL-17 (130,184). To investigate whether the action of T_H17 cells extends beyond the proinflammatory influence of IL-17, we explored the possibility that T_H17 cells might express cytolytic molecules and

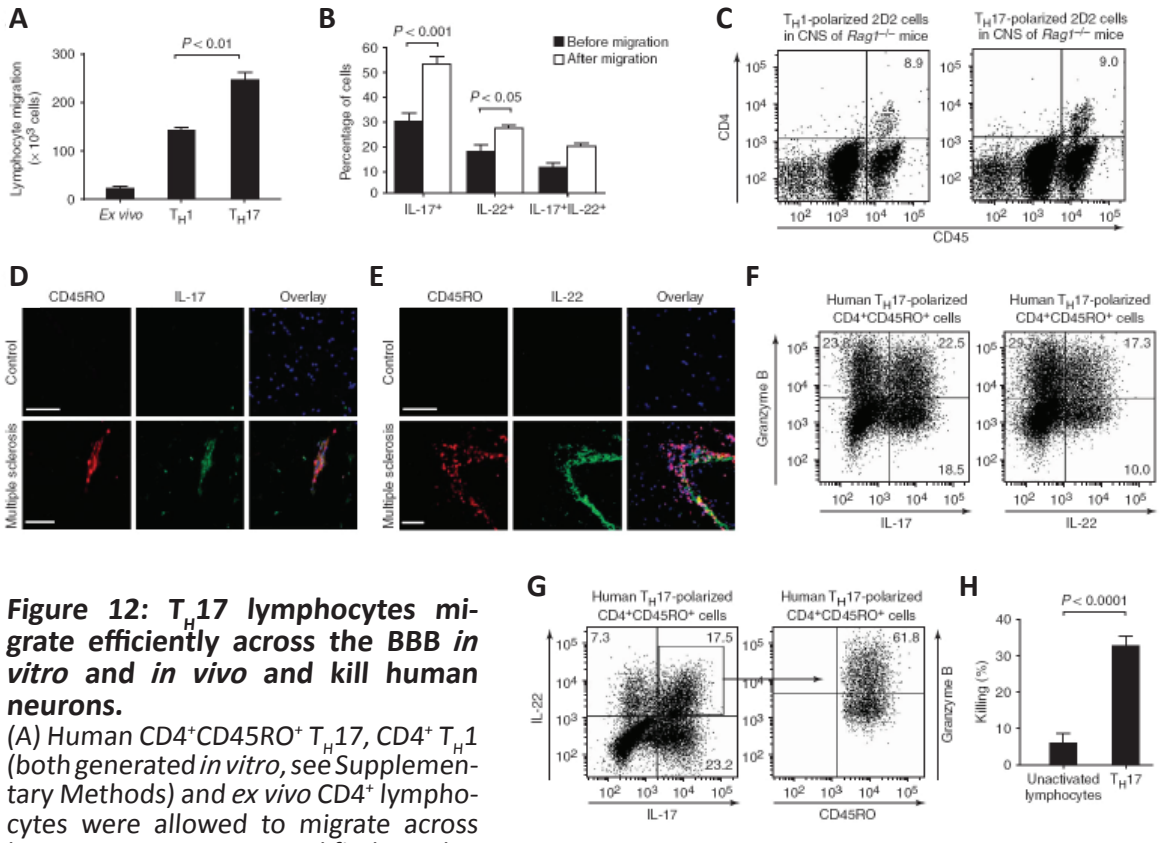


Figure 12: T_H17 lymphocytes migrate efficiently across the BBB in vitro and in vivo and kill human neurons.

(A) Human CD4⁺CD45RO⁺ T_H17, CD4⁺ T_H1 (both generated in vitro, see Supplementary Methods) and ex vivo CD4⁺ lymphocytes were allowed to migrate across human BBB-ECs in a modified Boyden chamber assay for 18 h. Significantly more T_H17 lymphocytes migrated than either T_H1 or ex vivo CD4⁺ lymphocytes. (B) CD4⁺CD45RO⁺ T_H17 lymphocytes were allowed to migrate across human BBB-ECs for 18 h. Cells were stained for IL-17 and IL-22 before and after migration. The cytokine profile revealed the preferential migration of IL-17⁺ and IL-22⁺ lymphocytes. (C) Immune cells from lymph nodes and spleen of $MOG_{35–55}$-immunized 2D2 mice were polarized toward T_H1 or T_H17 and transferred to Rag1$^{-/-}$ mice, and CD45^{hi}CD4⁺ lymphocytes were isolated from the CNS 7 d after transfer. Shown is a representative flow cytometry dot plot of CNS cell content from Rag1$^{-/-}$ mice injected with either T_H1- (left) or T_H17-polarized (right) 2D2 lymphocytes (n = 4 mice per group). (D) Human CNS postmortem material from unaffected individuals (control, non-inflamed, above) and heavily infiltrated CNS material from individuals with multiple sclerosis (below) were immunostained for CD45RO (red), IL-17 (green) and nuclear stain TO-PRO3 (blue). Confocal microscopy imaging confirmed the presence of IL-17⁺CD45RO⁺ cells (yellow) in infiltrated multiple sclerosis lesions but not in control CNS. Bar, 75 µm. (E) Similarly, IL-22⁺CD45RO⁺ staining was observed in multiple sclerosis lesions, but not in control CNS material. (F) Human CD4⁺CD45RO⁺ T_H17–polarized lymphocytes were stained for CD45RO, IL-17, IL-22 and granzyme B. Both IL-17– and IL-22–producing lymphocytes expressed granzyme B (22.5% and 17.3%, respectively). (G) More than 60% of IL-17⁺IL-22⁺ lymphocytes highly expressed granzyme B. Granzyme A and perforin were not detected in T_H17 cells, whether or not these cells produced IL-22 (data not shown). (H) The cytotoxic activity of T_H17 lymphocytes was assessed using neuron-enriched cultures obtained from human fetal CNS material and compared to that of unactivated T lymphocytes. All data shown are representative of the mean ±s.e.m. of three independent experiments.

therefore analyzed T_H17 cells for the expression of perforin, granzyme A and B. Notably, whereas virtually no *ex vivo* CD4⁺CD45RO⁺ cells produced cytolytic enzymes (data not shown), granzyme B was expressed in as many as 22.5% of IL-17–producing CD4⁺CD45RO⁺ cells and 17.3% of IL-22⁺ lymphocytes after 6 d of culture with IL-23 (**Fig. 12 F**). Even more striking is that 60% of cells coexpressing IL-17 and IL-22 also expressed granzyme B (**Fig.**

12 G). We therefore tested the capacity of granzyme B^+ T_H17 cells to kill human fetal neuron–enriched cultures and found that they showed considerable cytolytic activity (32.83 ±2.54%) as compared to unactivated T lymphocytes (6.15 ±2.37%) (**Fig. 12 H**, $P < 0.0001$, $n = 3$).

We next analyzed IL-17 receptor (IL-17R) and IL-22 receptor (IL-22R) expression on human BBB-ECs and investigated whether IL-17 and IL-22 influence BBB integrity. IL-17R and IL-22R were detected on the surface of a subset of human BBB-ECs in primary culture (**Fig. 13 A**, 23% of IL-17R$^+$ and 16% of IL-22R$^+$). *In situ*, however, IL-17R and IL-22R were undetectable in CNS material from subjects without multiple sclerosis. However, both receptors were strongly expressed on CNS vessels within heavily infiltrated multiple sclerosis lesions, colocalized with caveolin-1, a marker of brain endothelial cells (**Fig. 13 B, C**).

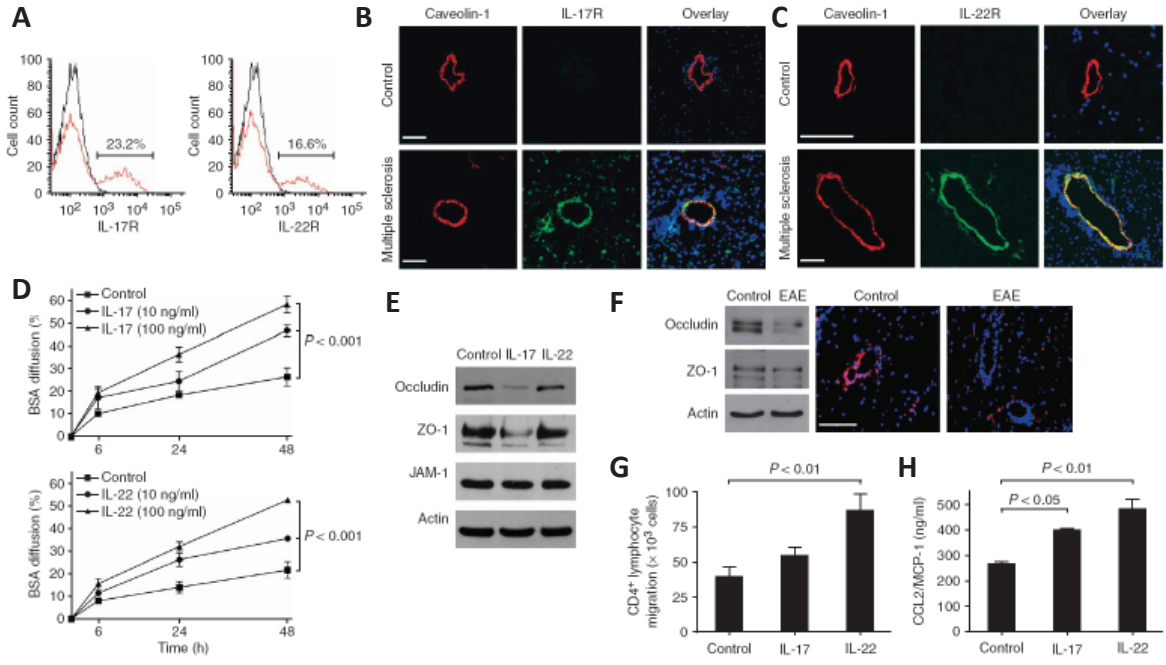
We further investigated whether brain endothelial IL-17R and IL-22R were functional and whether IL-17 and IL-22 could affect BBB permeability. Addition of 10 ng/ml of IL-17 or IL-22 to monolayers of human BBB-ECs induced a marked and sustained increase in the diffusion of fluorescence-labeled BSA (**Fig. 13 D**). This effect was dose dependent, reached a plateau at 100 ng/ml and coincided, for IL-17, with a decrease in the expression of occludin and zonula occludens (ZO)-1, two important tight junction–associated molecules (**Fig. 13 E**). A similar reduction of occludin and to a lesser extent ZO-1, expression was demonstrated by western blotting in spinal cord homogenates from EAE mice (**Fig. 13 F**). *In situ* staining further confirmed a decrease in ZO-1 immunoreactivity in cerebellar lesions of MOG-immunized mice (**Fig. 13 F**) These results are in line with our recent data showing a disruption of tight-junction proteins in highly infiltrated vessels of multiple sclerosis lesions (185). The exact mechanism mediating IL-22–induced BBB permeability remains uncertain, however.

We next explored the capacity of IL-17 and IL-22 to modulate lymphocyte migration across human BBB-ECs and found that IL-17 and IL-22 promote transmigration of human *ex vivo* CD4$^+$ lymphocytes (**Fig. 13 G**), most likely through enhanced BBB-EC–mediated secretion of CCL2 (or MCP-1) (**Fig. 13 H**). IL-17 also induced IL-6 and CXCL8 (or IL-8) expression by BBB-ECs, whereas expression of TGF-β, ICAM-1, VCAM-1, CCL5 (or RANTES) and CXCL10 (or IP-10) remained unaffected (data not shown). Taken together, these results strongly suggest that T_H17 cells, through the action of IL-17 and IL-22, play a unique role in permeabilizing the human BBB both to soluble molecules and to circulating CD4$^+$ lymphocytes.

Our study further refines the phenotype of human T_H17 lymphocytes as cells coexpressing IL-17, IL-22 and granzyme B and provides strong evidence that IL-17 and IL-22 induce a breach in the BBB and promote the recruitment of additional CD4$^+$ lymphocytes. Although IL-22 has the potential to influence the ability of T_H17 lymphocytes to gain access to the CNS, *in vivo* evidence indicates that IL-22 does not directly affect their encephalitogenicity

(B.B., personal communication). We postulate that T_H17 cells produce multiple mediators contributing to their highly encephalitogenic potential, among them cytolytic enzymes such as granzyme B.

Figure 13: IL-17 and IL-22 receptors are expressed on human brain endothelium, and their activation permeabilizes the BBB.
(A) Unactivated human BBB-ECs grown in primary culture were stained for IL-17R and IL-22R, revealing their expression on the surface of 23.2% and 16.6% of BBB-ECs, respectively. (B) Human CNS postmortem material from unaffected individuals (control, non-inflamed, above) and heavily infiltrated CNS material from individuals with multiple sclerosis (below) were immunostained for IL-17R (green), caveolin-1 (red) and nuclear stain TO-PRO3 (blue). Confocal microscopy imaging confirmed the expression of IL-17R on caveolin-1⁺ endothelium in inflamed CNS material. IL-17R expression was undetectable in control CNS material. Bar, 75 µm. (C) Similarly, IL-22R staining was observed on endothelial cells in multiple sclerosis lesions, but not in controls. (D) Human BBB-ECs were grown in Boyden chambers and treated with IL-17 (top) or IL-22 (bottom). Permeability of the monolayers was monitored with fluorescent BSA, showing that BBB-EC monolayer permeability increased after treatment with either IL-17 or IL-22. (E) Western blot for the tight-junction proteins occludin, ZO-1 and junction adhesion molecule (JAM)-1 from human BBB-ECs revealed disruption of occludin and ZO-1 by IL-17 (100 ng/ml, 18 h). (F) Western blot for tight-junction proteins in spinal cord homogenates of $MOG_{35–55}$-immunized EAE mice revealed a similar reduction in occludin and ZO-1. In situ immunostaining for ZO-1 (red) and nuclear stain TO-PRO3 (blue) in normal-appearing cerebellar white matter (control) and in infiltrated and demyelinated cerebellar lesions from C57BL/6 mice immunized with $MOG_{35–55}$ (EAE, grade 4). Confocal microscopy imaging confirmed disruption of ZO-1 around infiltrated vessels. Bar, 75 µm. (G) Freshly isolated peripheral blood human CD4⁺ lymphocytes were allowed to migrate for 18 h across IL-17– (100 ng/ml) or IL-22–treated (100 ng/ml) human BBB-ECs. Both cytokines promoted migration of human ex vivo CD4⁺ lymphocytes across human BBB-ECs, as compared to control. (H) CCL2 (or MCP-1) secretion by human BBB-ECs was assessed by ELISA in untreated and IL-17– or IL-22–treated cultures (100 ng/ml, 18 h). Both IL-17 and IL-22 upregulate CCL2 secretion by human BBB-ECs. All data shown represent the mean ±s.e.m. from three independent experiments performed in triplicate.



Materials and Methods

In vitro T_H polarization

We isolated mononuclear cells from peripheral blood of healthy human donors by density gradient centrifugation on Ficoll-Hypaque (GE Healthcare). We purified human $CD14^+$ monocytes, $CD4^+$ T lymphocytes and memory ($CD4^+CD45RO^+$) T lymphocytes by magnetic sorting (Miltenyi Biotec) according to the manufacturer's instructions. The cell purity was consistently >97%, as determined by flow cytometry. T cells (1×10^6 cells/ml) were cultured with autologous monocytes as antigen-presenting cells, at a two to one ratio and stimulated with CD3-specific antibody (2.5 µg/ml, clone OKT3, eBioscience) in RPMI 1640 medium supplemented with 5% human serum, 2 mM L-glutamine and antibiotics (Sigma). For T_H1 polarization, we added recombinant human IL-12 (10 ng/ml) and antibody to human IL-4 (5 µg/ml, clone 3007), whereas for T_H17 polarization, T cells were cultured in the presence of recombinant human IL-23 (10 ng/ml) as well as with neutralizing antibodies against IFN-γ (5 µg/ml, clone K3.53) and against IL-4. Recombinant cytokines and antibodies were purchased from R&D Systems. We harvested cells on d 6 for cytokine determination using commercially available ELISA kits for IFN-γ (BD Biosciences), IL-17 (Biosource) and IL-22 (R&D Systems) following the manufacturer's instructions. Levels of IL-17 and IL-22 were consistently higher in IL-23-stimulated lymphocytes, as compared to T_H1 and *ex vivo* lymphocytes (data not shown).

Intracellular cytokine staining and flow cytometry

After 6 d in culture under T_H1- or T_H17-biased conditions, we stimulated the cells 6 h with phorbol 12-myristate 13-acetate (20 ng/ml) and Ionomycin (1 µg/ml) in the presence of brefeldin A (2 µg/ml) (all from Sigma). We first stained cells for surface antigens CD3 and CD45RO then fixed and permeabilized them in 4% (w/v) paraformaldehyde containing 1% saponin. Subsequently, we added antibodies specific for human IL-17 (Biosource), IL-22 (R&D Systems), perforin, granzyme A (BD Biosciences) and granzyme B (Caltag Laboratories). IL-17R and IL-22R expression on BBB-ECs was detected using unconjugated antibodies (R&D
Systems) incubated with phycoerythrin-conjugated goat antibody specific to immunoglobulins (BD Biosciences). All staining assays were performed with the appropriate matched isotype control. Samples were acquired on a BD Biosciences LSR II flow cytometer and analyzed using BD FACSDiva Software.

BBB-EC isolation & culture.

We isolated BBB-ECs from CNS tissue specimens of temporal lobe resections from young adults undergoing surgery for the treatment of intractable epilepsy, as previously described (181,186). Informed consent and ethic approval were given prior to surgery (Comité d'Evaluation de la Recherche du CHUM, ethic approval number HD04.046). Cultures express endothelial markers factor VIII, Ulex Agglutenens Europaensis-1 binding sites and antigen HT-7 until passage seven to eight. No immune reactivity with β-tubulin, α-myosin or glial fibrillary acidic protein (GFAP) could be detected, confirming the absence of contaminating smooth muscle cells or astrocytes.

Migration assays.

Migration assays were performed on a 24-well plate modified Boyden chamber as previously described (181,182). In brief, we seeded 3×10^4 human brain ECs on top of a gelatin-coated 3 µm pore size membrane in EC culture media supplemented with 40% (v/v) astrocyte-conditioned media, shown to induce and maintain BBB characteristics *in vitro*. After 3 d of culture, the ECs had formed a confluent monolayer. At that point, when applicable, cells were treated for 24 h with 100 ng/ml of recombinant human IL-17 or IL-22 (R&D Systems). The next day, ECs were washed and a suspension of 1×10^6 T lymphocytes/ml was loaded in the upper chamber. We assessed the ability of both T_H1 and T_H17 lymphocyte subsets to cross the monolayer by counting the absolute number of cells that transmigrated to the lower chamber after 18 h, out of the 1×10^6 cells initially loaded in the upper chamber. All migration data shown represent at least three independent experiments performed in triplicate.

Permeability assays.

We performed the permeability assays on the *in vitro* model of the human BBB, as described for the migration experiments. We treated BBB-ECs on d 3 with 10 ng/ml or 100 ng/ml of recombinant human IL-17 or IL-22. After 24 h, we replaced the media and applied 50 µg/ml of fluorescein isothiocyanate-labeled BSA (Invitrogen) to the upper chamber. Fifty µl samples
were taken from the upper and lower chambers at different time points over a 3 d period and the fluorescence intensity in these samples was measured using a FL600 microplate fluorescent reader (Biotek). The diffusion rate, a measure of the permeability of BBB-ECs, was expressed as a percentage and calculated as follows: [BSA lower chamber] × 100 / [BSA upper chamber]. Each experiment was repeated at least three times in triplicate to ensure reproducibility.

Immunohistofluorescence staining for IL-17, IL-22, IL-17R and IL-22R.

Frozen CNS material from healthy donors (non-neurological disease controls) and MS subjects was obtained after autopsy. Ten µm sections were cut, fixed for 20 min in 4% paraformaldehyde and permeabilized with 1% triton X-100 for 5 min. Sections were blocked in HHG (1 mM HEPES, 2% horse serum, 10% goat serum in HBSS, Sigma) plus 0.5% triton X-100 for 1 h at room temperature, followed by overnight incubation at 4 °C with mouse antibodies specific to human IL-17 (1/20, eBiosciences) or human IL-22 (1/20, R&D Systems) and allophycocyanin-conjugated mouse antibody specific to human CD45RO (1/10, BD Biosciences). For the receptors, we used goat primary antibodies against human IL-17R and human IL-22R (1/20, R&D Systems) and rabbit antibody specific to human caveolin-1 (1/50, Santa Cruz). After several washes, IL-17, IL-22, IL-17R and IL-22R stains were amplified with biotin-conjugated goat antibody specific to mouse immunoglobulins (1/300) followed by streptavidin-fluorescein isothiocyanate (1/300 for IL-17 and IL-22, 1/1,000 for IL-17R and IL-22R, for 30 min at room temperature). CD45RO staining was visualized with allophycocyanin-specific antibody raised in rabbit (10 µg/ml, Biomeda) followed by Cy3-coupled goat antibody specific to rabbit immunoglobulins (1/300, Jackson ImmunoResearch). Caveolin-1 staining was visualized with Cy3-coupled goat antibody specific to rabbit immunoglobulins (1/400). Nuclei were stained with TOPRO3 (1/300 in phosphate buffered saline for 15 min, pre-treatment with 100 µg/ml RNase A for 30 min, Molecular Probes). All control staining were performed omitting the primary antibody, in which case no immunopositive cells could be detected. Staining was visualized using a Leica SP5 confocal microscope and analyzed with Leica LAS AF Software.

Cytotoxicity assays of fetal human neuron-enriched cultures

Brain tissue from human fetuses of 16–20 weeks gestational age were obtained following therapeutic abortion according to guidelines approved by the Research and Ethic Committee, University of Alberta and informed consent was given by the parents. Neuron-enriched cultures (>90% purity) were prepared as previously described (187) and 1×10^5 cells were plated in individual chambers 72 h prior to the killing assay. 1×10^5 unactivated lymphocytes and IL-23-stimulated T_H17 cells were added to the neurons for 24 h, fixed with 4% paraformaldehyde and labeled with a monoclonal antibody to microtubule-associated protein (MAP)-2 (1/1,000, Sigma). Neuron (MAP-2^+ cells) survival was evaluated counting six random fields in each well using the 40 × microscope objective. For every condition in each set of experiments, four wells were counted to obtain the mean number of neurons remaining in culture. In figure 1 h, data are expressed as the percentage of killing and represent 100% – percentage of survival, from three independent experiments.

Animals

Rag1$^{-/-}$ mice were purchased from The Jackson Laboratory and were bred under specific pathogen-free conditions. The 2D2 (MOG-TCR-Tg) mice were provided by Dr. V.K. Kuchroo (Harvard Medical School). Animal experiments and breeding were approved by the Swiss Veterinary Office (69-200370-2003).

Adoptive transfer of T_H1 and T_H17 cells.

2D2 mice were immunized subcutaneously with 200 µg of $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK, obtained from GenScript) emulsified in complete Freund adjuvant (Difco). Seven days later, mice were sacrificed using CO_2. Spleens, axillary and inguinal lymphnodes were isolated, treated with 0.5 mg/ml DNase and 1 mg/ml Liberase (Roche) for 30 min at 37 °C and homogenized. The cells were cultured for 4 d in RPMI 1640 supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 ug/ml streptomycin (Gibco) in the presence of 15 µg/ml of $MOG_{35–55}$ peptide, 10 µg/ml PolyI:C (Fluka) and either 2.5 ng/ml of recombinant IL-12 (PeproTech) for the promotion of T_H1 cells or 10 ng/ml of recombinant IL-23 (PeproTech) for the promotion of T_H17 cells. Cells were harvested, washed and injected into recipient mice (10×10^6 cells/mouse). Animals received 200 ng pertussis toxin (Sigma) intraperitoneally at the time of transfer and 48 h later.

Flow cytometry.

Mice were sacrificed 7 or 9 d after adoptive transfer using CO_2 and spinal cords and brains were isolated, treated with 0.5 mg/ml DNase and 1mg/ml Liberase for 30 min at 37 °C, homogenized and strained through a nylon filter with a pore size of 100 µm (Fisher). After centrifugation, cells were resuspended in 30% Percoll (Pharmacia) and were centrifuged at 18,500 *g* for 30 min at 4 °C. Viable immune cells were collected and washed extensively before being stained. For flow cytometry, antibodies (all purchased from BD Pharmingen) were incubated with cells for 20 min at 4 °C and then cells were analyzed with a FACSCanto (BD Pharmingen) and FACSDiva software. Postacquisition analysis was performed with FACSDiva Software (BD Pharmingen).

Western blots for tight junction proteins

For western blots for ZO-1, occludin, JAM-1 and actin, we lysed BBB-ECs in NP-40 buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl2 and 0.5% NP-40) supplemented with protease inhibitors. Fifty µg of total protein were electrophoresed on a 10% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to a polyvinylidene fluoride

membrane (Biorad) and blocked for 1 h at room temperature in 5% milk. We incubated the membranes overnight with rabbit antibody specific to human occludin, rabbit antibody specific to human ZO-1 (1/250, Zymed), mouse antibody specific to human JAM-1 (1/500, BD Bioscience) or mouse antibody specific to human actin (1/1,000, 1 h at room temperature, MP Biomedicals) followed by an incubation with horseradish peroxidase (HRP)-conjugated goat antibody specific to rabbit immunoglobulins or rabbit antibody specific to mouse immunoglobulins (1/1,000, 1 h at room temperature, DakoCytomation). Specific binding was visualized using the ECL system (Amersham Biosciences).

Immunohistofluorescence staining for ZO-1

Cerebellum tissue samples from MOG-immunized C57BL/6 mice at the peak phase of EAE were frozen in O.C.T. and cut into 10 µm sections. Sections were permeabilized and blocked as described above and immunostained overnight at 4 °C with ZO-1-specific antibody (1/50, Zymed). ZO-1 signal was amplified with biotin-conjugated goat antibody specific to rabbit immunoglobulins (1/300) followed by streptavidin-Cy3 (1/300) antibody. Nuclei were visualized with TO-PRO3 (1/300). ZO-1 staining in highly infiltrated vessels was compared to the signal in normal-appearing white matter of the same animals.

ELISA

BBB-ECs were treated with recombinant human IL-17 (100 ng/ml), recombinant human IL-22 (100 ng/ml) or both. We collected supernatants at 24, 48 and 72 h and assessed for secretion of CCL2 (or MCP-1) by ELISA, using a commercially available kit from BD Biosciences. ELISAs were carried out following the manufacturer's instructions. Standards and samples were run in duplicate.

Statistical analysis

Migration data are expressed as the mean ± s.e.m. For cytokine level comparisons and migration assays, one-way analysis of variance (ANOVA) was performed followed by Bonferroni multiple comparison post-test. Comparison of IL-17 and IL-22 treatments in permeability studies was done using two-way ANOVA, followed by the Bonferroni post-test. Differences between groups were considered significant when $P < 0.05$.

Author contributions

H.K. conducted most of the experiments, K.K. performed and analyzed animal studies, I.I. and A.D.-D. contributed to immunostaining and *in vitro* protocols, R.C. assisted with confocal microscopy and performed some EAE experiments, M.B. assisted with BBB-EC isolation and culture, F.G. performed the killing assay, N.A. provided critical input on data analysis, B.B. designed and supervised the animal studies, H.K. and A.P. designed the study, analyzed the data and wrote the manuscript, A.P. secured the funding.

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Discussion

T_H17 cells in autoimmunity (current view)

While T_H1 cells were long suspected to be the major pathogenic population in autoimmune diseases such as EAE, the discovery that IL-23 and not the T_H1 inducing cytokines IL-12 and IL-18 is vital for EAE development initiated a major paradigm shift with regards to the role of T_H1 cells in inflammation (78,79,99,154). Langrish *et al.* could show that IL-23 induces the secretion of IL-17A by effector T cells (101) and IL-17 expression by T cells correlates closely with an autoimmune pathogenic phenotype and this polarization pattern was termed T_H17 (14,15,188). While studying IL-23 under various inflammatory conditions initially led to the discovery of T_H17 cells, it was later found that the cytokines TGF-β plus IL-6 induce differentiation of T_H17 cells, IL-21 amplifies the frequency of T_H17 cells and IL-23 stabilizes the phenotype of previously differentiated T_H17 cells (155). However, the exact role and function of these cytokines – and IL-23 in particular – in inducing and maintaining this phenotype remains a subject of debate (130).

The importance of the T_H17 subset in autoimmune diseases was first demonstrated in mice deficient in IL-23. These mice had similar numbers of IFN-γ-producing T cells as wt mice but showed a dramatic decrease in IL-17-producing T cells and were resistant to the development of EAE and CIA (99,169).

T_H17 cells are now closely associated with a number of inflammatory autoimmune diseases such as RA, MS and psoriasis and are widely recognized as the pathogenic population in autoimmunity.

However, in spite of this close correlation (99,101,169), several questions regarding the biological relevance of T_H17 cytokines and their function, especially in autoimmune diseases, remain to be answered.

T_H17 cytokines in autoimmunity

IL-17A in EAE

Transgenic targeting of IL-17A to T cells does not alter the pathogenesis and clinical development of MOG-induced EAE.

To investigate the impact of IL-17A expressed by CNS invading T cells on the pathogenesis of EAE, we generated a mouse conditionally overexpressing IL-17A in T cells. Its $CD4^+$ T cells produced highly elevated levels of IL-17A but this did not result in an overt phenotype

neither in the macroscopic constitution of the mice nor in the cellular compositions of thymus, spleen and lymph nodes. When we induced EAE in these mice, we found that IL-17A overexpression in T cells had no effect on the on the development of disease or cell infiltration into the CNS and no significant alterations were observed with respect to IFN-γ expressing cells. However, despite the indistinguishable clinical scores, CNS samples from mice overexpressing IL-17A did show a clear and highly significant increase in IL-17A$^+$ T cells compared to CNS samples from mice in which the IL-17A overexpression was not induced. Therefore, an increase in T cell derived IL-17A in the inflamed CNS during MOG-induced EAE does not result in an appreciable alteration of the disease course. Considering various reports describing the upregulation of IL-17A expression in CNS lesions of MS patients (102,129), this result was surprising but cannot yet rule out IL-17A as a crucial cytokine in CNS inflammation as it could be that low IL-17A concentrations are sufficient to exert its function. Therefore, the IL-17A levels present under non overexpressing conditions might suffice and the IL-17A response might be elicited once a threshold is reached. Investigating the situation under IL-17A deficient conditions could clarify this issue.

IL-17A function is redundant in the development of EAE

To improve the understanding of the role of the T_H17 effector type in autoimmunity we next analyzed the impact of loss of IL-17A on the development of EAE. Despite the close association of IL-17A with autoimmune inflammation of the CNS, the loss of IL-17A only marginally impeded the induction of the disease, which is similar to the observations made by Iwakura and colleagues (130). The function of IL-17A also seems to be redundant in other models of autoimmunity such as experimental autoimmune uveoretinitis (EAU) (163). Even though IL-17A deficient mice are susceptible to EAE, they do show a delayed onset and reduced severity and IL-17A deficient mice or mice treated with an IL-17R antagonist have been reported to be resistant to the development of other autoimmune diseases like adjuvant-induced arthritis (125,128). A function of IL-17A in autoimmunity can therefore not be dismissed altogether and might depend on the specific disease model and target tissue. In support of this assumption, we observed early signs of skin inflammation and ultimately a developmental retardation clearly visible from P4-6 on in mice ectopically overexpressing IL-17A. This finding matches the reported role of IL-17A in inflammatory skin diseases like psoriasis (102).

The non-essential role of IL-17A in EAE development, however, could also result from an involvement of other T_H17 associated factors. To investigate the exact nature of the T_H17 response in the immunized IL-17A deficient mice, we analyzed their cytokine profile upon *in vitro* restimulation with MOG peptide under T_H17 polarizing conditions and found IL-17F to be consistently and drastically upregulated in the lymphocytes of IL-17A deficient mice.

This specific increase of one T_H17 cytokine together with the high degree of homology between IL-17A and IL-17F and the shared receptor subunits, lets it seems feasible that these two cytokines exert similar functions and suggests the possibility of a compensatory role of IL-17F in disease development.

IL-17F in EAE

IL-17F is expressed by T_H17 cells and is abundant in the inflamed CNS

A screen performed by quantitative RT-PCR of cerebellum of diseased mice revealed that IL-17F, like IL-17A, is highly expressed in the inflamed CNS as compared to cerebellum of healthy controls. To identify the source of IL-17F in the context of EAE we restimulated *in vivo* primed splenocytes *in vitro* with MOG peptide under T_H17 polarizing conditions and found that IL-17F expression is restricted to MOG-responsive and potentially encephalitogenic $CD4^+$ T_H17 cells.

The finding that both cytokines are present in the inflamed CNS and mark the highly pathogenic T_H17 population that has been associated with autoreactive lesions suggests that IL-17F is an encephalitogenic cytokine with functional relevance in CNS autoimmune inflammation.

Generation and analysis of the IL-17F deficient mice

To ultimately determine whether IL-17F contributes to the development of EAE, we generated IL-17F deficient mice. Against our expectations, the lack of IL-17F did not have any impact on the clinical EAE development with regards to the day of disease onset, maximum score or incidence. Detailed analysis of CNS-infiltrating cells revealed no change in cell numbers or composition of CNS invading leukocytes.

The full susceptibility of $IL\text{-}17F^{-/-}$ mice to EAE could have resulted from a compensatory increase in the production of IL-17A. However, $IL\text{-}17F^{-/-}$ lymphocytes showed a decrease in the production and frequency of IL-17A secreting cells, letting a compensatory effect by IL-17A seem unlikely. To definitively rule out a reciprocal compensation of IL-17A and IL-17F one had to investigate mice lacking both genes. Unfortunately, the close proximity of the loci of these two cytokines makes it very unlikely to obtain double-deficient mice by interbreeding (ca. 44 Kb in between the two genes) and therefore an approach using neutralizing antibodies against one of the cytokines in the reciprocal knockout mouse could clarify this issue.

The decreased IL-17A secretion in IL-17F targeted T cells was also observed in a different IL-17F deficient mouse strain, generated by insertion of a transcriptional stop into exon 2 (167). A possible explanation is that either the gene-targeting of IL-17F interferes with the promoter/enhancer activity of IL-17A or that IL-17F exerts some kind of regulatory effect

on IL-17A production. In any case, the fact that IL-17F$^{-/-}$ mice show drastically reduced in IL-17A levels strongly supports the assumption that neither IL-17A nor IL-17F, either individually or in combination, are essential for the development of autoimmune CNS inflammation.

IL-22 in EAE

IL-23 induces IL-22 gene expression in T_H17 cells

To identify gene transcripts driven by IL-23, we devised two reciprocal approaches for whole genome transcriptomics. We compared gene-expression induced by IL-23 stimulation with those absent in Ag-driven IL-23 deficient lymphocytes. In the first approach, genes more than fourfold upregulated by IL-23 were identified by stimulating splenocytes obtained from an unmanipulated mouse with recombinant IL-23 or IL-12 as a control. In the second approach, we immunized wt, IL-12p35$^{-/-}$ and IL-12/23p40$^{-/-}$ mice with KLH and harvested lymphocytes and re-challenged them *in vitro* prior to harvesting the mRNA for microchip analysis. We used IL-12 as a control firstly, because IL-12-induced gene expression is well characterized and secondly to eliminate IL-12-induced target genes from our analysis. To verify that the stimulation worked properly, we first analyzed the expression profile of genes known to be induced by IL-12 or IL-23, respectively. For IL-12 stimulated cells we analyzed cells for IFN-γ expression, for IL-23 stimulation we measured IL-17 expression, as IFN-γ (189) and IL-17 (97) are known to be induced by IL-12 or IL-23, respectively. By combining both data sets, we found IL-22 to be specifically and strongly induced by IL-23. We could verify that other than IL-23, none of an array of different stimuli used elicited significant levels of IL-22 expression in splenocytes. Previously, Dumoutier *et al.* could detect IL-22 gene expression after stimulation with IL-9 (131) – but for these experiments a lymphoma cell line was used and the different finding could therefore be explained by the often altered gene expression profile of cancer cells. Also, they observed IL-22 expression in splenocytes upon ConA activation, which was not inhibited after IL-9 neutralization already suggesting that other cytokines mediate IL-22 induction. Furthermore, IL-6 has been shown to be sufficient for the induction of IL-22 from naïve CD4$^+$ T cells by another group (140). For these experiments, FACS sorted naïve CD4$^+$ cells have been used and additionally stimulated with anti-CD3 and anti-CD28 antibodies which supply an unspecific and very strong stimulus to T cells. Therefore, one cannot directly compare this finding to ours. In our model, IL-23 was clearly the only stimulus able to induce IL-22 production *in vitro*. This observation is further supported by other *in vivo* data: Although both IL-6 and IL-23 stimulate IL-22 production *in vitro* (140), only IL-23 seems to be indispensable *in vivo* for IL-22 induction under several infectious or autoimmune-disease conditions (141,142). Also, IL-6 is not essential for IL-22 induction in ConA-induced hepatitis and *C. rodentium*

infection models (141,150).

When we stimulated purified $CD4^+$ as well as $CD8^+$ T cells obtained from OT-II and OT-I mice, respectively, with cognate peptide pulsed DCs we found that only TcR Tg $CD4^+$ T cells made IL-22, verifying the notion that T_H cells and not CTLs are the main source of IL-22. It was, however, recently reported that IL-23 induces IL-22 production also from $CD8^+$ T cells and γ/δ T cells, as well as monocytes and $CD11c^+$ DCs (140,141). This is not in line with our finding and also contradicts a previous report showing that monocytes do not express IL-22 after LPS stimulation in which case IL-23 should also be present (137). However, $CD8^+$ T cells as well as monocytes and DCs do express the IL-23R (91,92) and could therefore potentially respond to its engagement with IL-22 production but it needs to be further elucidated weather they do so only under certain conditions or weather other reasons are responsible for the differing results regarding the IL-22 producing cell type.

In agreement with Liang *et al.*, we found IL-22 to be highly expressed by the T_H17 cell subtype (139), which is closely associated with effector functions in autoimmunity (170) suggesting that IL-22 could potentially serve a pathogenic function during EAE.

IL-22 is expressed by CNS invading encephalitogenic T_H cells

We determined the induction of IL-22 expression in a more physiologic manner in response to cognate Ag presented by DCs. MOG-reactive T cells clearly expressed high levels of IL-22 and also IL-17A after encounter with their cognate antigen. This response was dependent on IL-23 as reduced levels of IL-22 and IL-17 were detectable when T cells were co-cultured with DCs obtained from p40IL-12/$23^{-/-}$ mice. Our findings together with data from a similar experiment reported by Liang *et al.* in which recombinant IL-23 was added during a recall assay further underline the importance of IL-23 for IL-22 induction (139). We analyzed the expression of IL-22 in mice with autoimmune inflammation and detected a significant production of IL-22 by encephalitogenic, CNS-infiltrating lymphocytes after re-encounter with their cognate MOG-antigen. Similar to IFN-γ and IL-17, IL-22 expression by CNS-infiltrating lymphocytes increased with disease severity.

To this end, all data hint towards IL-22 expression actually marking a highly pathogenic and proinflammatory population of autoaggressive T cells as recently claimed by Bettelli *et al.* (170), implicating IL-22 to exert a pathogenic function during EAE.

IL-22 deficient mice are fully susceptible to EAE

In order to determine whether IL-22 actually contributes to the development of EAE or whether the crisp correlation between IL-22 expression and encephalitogenicity is only an epiphenomenon, we generated IL-$22^{-/-}$ mice by gene-targeting. The genomes of the C57/Bl6 mouse strain possess two IL-22 genes, IL-22 α and β. The IL-22 β gene contains several

single nucleotide changes and a deletion covering the first noncoding exon and a segment of a putative promoter, already suggesting that the IL-22β gene may not be expressed. We verified the absence of IL-22 by genomic PCR, RT-PCR using primers amplifying both IL-22 α and β and ELISA in the IL-22$^{-/-}$ mouse, proofing that the knockout of the IL-22α gene was successful and that the IL-22β gene is indeed not expressed in the C57/B6 mouse strain.
IL-22 has been reported to have a role in proliferation of some cell types such as keratinocytes or breast cancer cells (145,190). IL-22$^{-/-}$ lymphocytes, however, behaved similar to wt cells after re-encounter of cognate Ag with regards to proliferating capacity as well as cytokine expression profile. Therefore, IL-22 deficiency does not impair lymphocyte function in general, which was expected due to the missing IL-22R expression on these cells (191,192).
When we induced EAE in the IL-22$^{-/-}$ mice, we discovered to our surprise that IL-22$^{-/-}$ mice develop EAE with the same severity, day of onset and clinical manifestations as wt mice. This finding clearly dismisses IL-22 as a major pathogenic player in the development of autoimmune CNS inflammation.

IL-22 in other disease models

The function of IL-22 in autoimmunity however cannot be dismissed altogether. Wolk *et al.* reported that elevated levels of IL-22 can be found in the blood of psoriatic patients and ear-skin acanthosis and inflammation induced by the application of IL-23 is slightly decreased when IL-22 is absent (140,145). Furthermore, an upregulation of IL-22 has been described in CD and UC, as well as in preclinical mouse IBD models or RA patients (135,148,193). In addition to its proinflammatory role, protective functions of IL-22 have also been described (123,149,150). In support of a potentially protective function of IL-22, we observed that IL-22$^{-/-}$ mice developed more severe disease in a model of allergic asthma with increased eosinophil invasion into the lung. The IL-22R is not expressed on eosinophils, but on epithelial cells of the respiratory tract. Therefore, it is likely that IL-22 exerts an indirect function on eosinophil recruitment through mediators secreted by epithelial cells in response to IL-22R engagement. Further proving a protective role of IL-22 in airway inflammation, administration of recombinant IL-22 attenuates the inflammatory response in the lung (Taube C., Kreymborg K., manuscript in preparation). Interestingly, IL-17F has been reported to be present in the airways of patients with allergic asthma and a mutation in the IL-17F gene was shown to be associated with human asthma and chronic obstructive pulmonary disease (158,194) which suggests that there might also be a role of IL-17F in asthma. Therefore, we will in the future also investigate the role of IL-17F in this disease by utilizing the IL-17F$^{-/-}$ mice in our model of allergic asthma.
In conclusion, IL-22 does not contribute to autoimmune inflammation of the CNS and

cannot be categorized as a cytokine with either clearly pathogenic and protective properties but its function seems to depend on the specific situations and target cells.

IL-22 and IL-17 mediated permeabilization of the BBB

We could detect IL-17R and IL-22R on the surface of a subset of human BBB epithelial cells in primary culture. *In situ*, however, IL-17R and IL-22R were undetectable in CNS material from subjects without MS but strongly expressed on CNS vessels within heavily infiltrated MS lesions. We could further show that IL-17 and IL-22 could affect BBB permeability since addition of these cytokines to monolayers of human BBB epithelial cells induced a marked and sustained increase in the diffusion of fluorescence-labeled BSA and for IL-17 it coincided with a decrease in the expression of occludin and ZO-1, two important tight junction–associated molecules. These results are in line with recent data showing a disruption of tight-junction proteins in highly infiltrated vessels of MS lesions (185). We also explored the capacity of IL-17 and IL-22 to modulate lymphocyte migration across human BBB epithelial cells and found that IL-17 and IL-22 promote transmigration of human *ex vivo* $CD4^+$ T cells, most likely through enhanced BBB epithelial cell mediated secretion of CCL2 (or MCP-1). Considering the recently reported ability of IL-17A to very efficiently induce chemokine production by lung epithelial cells it could very well be that IL-17 and IL-22 exert a similar effect on BBB epithelial cells (119). These results strongly suggest that T_H17 cells, through the action of IL-17 and IL-22, play a unique role in permeabilizing the human BBB both to soluble molecules and to circulating $CD4^+$ T cells.

However, given the IL-17R and IL-22R are only expressed in the inflamed CNS and not under healthy conditions, IL-17 and IL-22 are unlikely to be responsible for the initial BBB breakdown. More likely, these cytokines contribute to further BBB permeabilization in an already inflamed environment – still not explaining the fundamental and crucial steps leading to CNS inflammation.

Furthermore, when we generated MOG-specific T_H1 and T_H17 lymphocytes from 2D2 mice *in vitro* and transferred these separately into $Rag1^{-/-}$ mice we found equal numbers of T_H cells in the CNS 7 d after transfer, regardless of whether donor cells were polarized into T_H1 or T_H17 cells. These data indicate, that high expression of IL-17 and IL-22 – as it is the case for T_H17 but not T_H1 cells – cannot be the only prerequisite to invade the CNS and further points toward the existence of additional factors essential in the process of CNS inflammation.

T_H17 function beyond IL-17A, IL-17F and IL-22

Evaluating all data generated by deletion of the T_H17 effector cytokines IL-17A, IL-17F and IL-22 or the induced overexpression of IL-17A it is clear that none of those T_H17 cytokines are key players in autoimmune disease like EAE.

Given the strong phenotype of IL-23 deficient mice in autoimmunity, it is likely that there are additional factors induced by IL-23 or thus far unidentified T_H17 cytokines or factors with crucial functions in autoimmune inflammation. This concept is also supported by the report of McGeachy *et al.*, who observed that IL-23 driven T_H cells, but not TGF-β/IL-6 driven T_H cells were encephalitogenic, regardless of their secretion of IL-17A (115).

Cytolytic enzymes

Employing an *in vitro* model of the human BBB using human brain-derived microvascular endothelial cells we found that human T_H17 lymphocytes migrated avidly across the BBB. We could detect numerous $CD45RO^+$ cells immunopositive for IL-17 or IL-22 in highly infiltrated MS lesions, but not in normal-appearing white matter or non-inflamed brain specimens. We observed that, whereas virtually no *ex vivo* $CD4^+CD45RO^+$ cells produced cytolytic enzymes, granzyme B was expressed by IL-17– or IL-22–producing $CD4^+CD45RO^+$ cells after 6 d of culture with IL-23. Strikingly, the cells coexpressing IL-17 and IL-22 were the major population expressing granzyme B. Granzyme B^+ T_H17 cells had the capacity to kill human fetal neuron–enriched cultures and showed considerable cytolytic activity as compared to unactivated T cells.

$CD4^+$ cells are typically known for their capacity to provide helper functions during an immune response and cytolytic activity was long thought to be limited to $CD8^+$ T cells. However, over the past two decades the ability of $CD4^+$ T cells to display cytotoxic potential has been reported in both mouse and human, although the existence and significance of $CD4^+$ CTLs have been disputed or disregarded (195). It has been suggested earlier, that $CD4^+$ CTLs use the perforin-dependent cytotoxic mechanism, rather than the Fas-dependent pathway and recent reports finally proofed that $CD4^+$ T cells are also able to perform perforin and granzyme-mediated killing and the *in vivo* existence and relevance of cytotoxic $CD4^+$ T cells has been established (20). Our observation regarding granzyme B expression by $CD4^+$ T cells further refines the phenotype of human T_H17 lymphocytes as cells coexpressing not only IL-17 and IL-22 but also additional mediators contributing to their encephalitogenic potential, among them cytolytic enzymes like granzyme B. Therefore, the action of T_H17 cells clearly extends beyond the proinflammatory influence of IL-17A, IL-17F and IL-22.

GM-CSF

In the whole genome transcriptomics approach we performed to reveal IL-23 induced gene transcripts, we identified IL-22 to be highly inducible by IL-23 but also further genes which expression was, albeit to a lesser degree, IL-23 dependent. Tnfrsf11b, Pkia and Pgcp-pending were upregulated upon IL-23 stimulation and Csf2, Dab2, Klra16, Hdgf and IL-1α were downregulated in the absence of IL-23. The IL-23 dependent gene expression was verified by real time PCR and of these potential candidate genes, we found only colony-stimulating factor 2 (Csf2) expression to be clearly upregulated upon stimulation with IL-23 as well as reduced in $p40^{-/-}$ lymphocytes. There was no significant difference in the expression of all the other genes we tested.

Colony-stimulating factor 2, better known as granulocyte macrophage colony-stimulating factor (GM-CSF), is the major regulator involved in the control of granulocyte and macrophage linage populations at all stages of maturation. Interestingly, GM-CSF plays a central role in maintaining chronic inflammation and its role in autoimmune-mediated demyelination has only recently been described. GM-$CSF^{-/-}$ mice are resistant to EAE, display decreased antigen-specific proliferation of splenocytes and fail to sustain immune cell infiltrates in the CNS, thus revealing key activities for GM-CSF in the development of inflammatory demyelinating lesions and control of migration and/or proliferation of leukocytes within the CNS (196). Furthermore, T cells expressing GM-CSF induce severe and chronic EAE (197). Ifergan *et al.* recently demonstrated that through secretion of TGF-β and GM-CSF, human BBB endothelial cells promote the differentiation of a subset of peripheral blood $CD14^+$ monocytes into $CD83^+$ (myeloid) DCs which express CD209 and secrete IL-12p70, TGF-β and IL-6, favoring the differentiation of distinct $CD4^+$ T cell populations into IFN-γ- or IL-17- secreting cells (198). Based on these data one could speculate a possible mechanism for the action of GM-CSF in mediating CNS inflammation being the recruitment of peripheral blood derived monocytes, as well as their differentiation into functional DCs, which have been proven to be essential in EAE pathogenesis (36). Taken together, GM-CSF might be an additional candidate for a thus far unidentified IL-23 dependent “encephalitogenicity-gene” potentially explaining the strong phenotype observed in IL-$23^{-/-}$ mice.

T_H17 cells in autoimmunity (reconsidered)

The discovery of the T_H17 lineage and the biological functions of its effector cytokines undoubtedly advanced our understanding of $CD4^+$ T cells in adaptive immunity. But despite the recent progress many issues particularly concerning their pathogenic role in autoimmunity remain inconclusive.

First of all, data obtained from experiments using *in vitro* differentiated T_H17 cells have to

be evaluated with great care since the protocols widely used for this purpose are highly artificial. They include the administration of unphysiologically high doses of a cohort of cytokines, factors and neutralizing antibodies that are under no circumstances present like this *in vivo*. Therefore, while these *in vitro* experiments help to gain insights into conditions ideal to polarize T cells toward a desired phenotype, they can only give a vague idea as to how these cells function *in vivo*. Furthermore, the conditions promoting human T_H17 cell differentiation are not universally established and how IL-6, TGF-β, IL-23 and other factors control T_H17 cells *in vivo* under various inflammatory conditions is still unclear. In fact, T cells are exposed to a complex cytokine milieu *in vivo* and display considerable plasticity. For example, many of the $CD4^+$ T cells isolated from an inflamed CNS during EAE do not exclusively belong to either the T_H1 or T_H17 T cell subsets as currently defined because they coproduce IL-17 and IFN-γ.

Strikingly, many studies claiming T_H17 cytokines to be the main driving force behind tissue inflammation are based on correlative relationships. For example, the decreased expression of IL-17A and IL-17F observed in IL-23- or IL-6-deficient mice, which are resistant to EAE (99,199-202), has led to an overhasty interpretation making these cytokines responsible for the observed phenotype though the restrained production of IL-17A and IL-17F much rather is a symptom than the cause of the EAE resistance. However, given the strong phenotype of IL-23 deficient mice in autoimmunity which cannot – as we have demonstrated here – be explained by so far identified T_H17 cytokines such as IL-17A, IL-17F and IL-22, it is likely that there are additional factors or mechanisms induced by IL-23 or thus far unidentified T_H17 cytokines driving autoimmune inflammation.

Finally, T_H17 cells and their effector cytokines can also have protective roles during inflammation (123,149,150) and the balance of their functions is not well understood during the processes of many autoimmune and infectious diseases.

Taken together, the wish to identify the one culprit in autoimmunity (together with facilitated publishing when including the right code word in line with the current trend) can lead to a simplistic black and white perception of the in fact very complex and reciprocal interplay of cells and factors inducing the development of autoimmune inflammation. This has lead to a biased interpretation of the role and function of T_H1 and T_H2 cells in the past and now puts the blame on T_H17 cells.

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Publications

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